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Europäisches Patent : **EP 0 656 786 B1**
Anmeldenummer : 93909679.8
Inhaber : Kelly, Graham Edmund, Northbridge, NSW 2063 (AU)
Titel : Use of Isoflavone Phyto-Oestrogen Extracts of Soy and Clover

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Gegen das oben bezeichnete Europäische Patent EP 0 656 786 B1 wird hiermit in vollem Umfang Einspruch erhoben und Widerruf des Patentes für alle Vertragsstaaten, für die es Wirkung hat, beantragt. Hilfsweise wird Antrag auf mündliche Verhandlung gestellt.

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Die Einspruchsgebühr in Höhe von € 610,00 soll von unserem laufenden Konto Nr. 28000966 abgebucht werden. Ein Abbuchungsauftrag für das Referat Kassen- und Rechnungswesen ist diesem Schreiben beigefügt.

1. Einspruchsgründe

Das Streitpatent ist gemäß Artikel 100 a) EPÜ nicht patentfähig, da es die Anforderungen an die erfinderische Tätigkeit (gemäß Artikel 56 EPÜ) nicht erfüllt. Des Weiteren ist gemäß Artikel 100 b) EPÜ die Erfindung nicht so deutlich und vollständig offenbart, dass ein Fachmann sie ausführen kann.

2. Entgegenhaltungen

Der Einspruch stützt sich auf folgende Druckschriften:

[D1] J. of Chromatography, 298 (1984) 175-182, J. Sachse, Quantitative Hochdruckflüssigkeitschromatographie von Isoflavonen in Rotklee

[D2] Biochemical Journal, Vol.58, 1954, 283-287, D.H. Curnow, Oestrogenic Activity of Subterranean Clover

[D3] Cancer Research 48, 6257-6261, November 1988, John M. Cassady et al, Use of Mammalian Cell Culture...Inhibition of Metabolism by Biochanin A, an Isoflavone from Trifolium pratense

[D4] Food Additives and Contaminants, 1985, Vol.2, No.2, 73-106, K.R. Price, G.R. Fenwick, Naturally occurring oestrogens in foods-A review

[D5] Reproductive Toxicology, Vol.3, pp.81-89, 1989, Rami S. Kaldas et al., Reproductive and General Metabolic Effects of Phytoestrogens in Mammals

[D6] Arzneimittelwirkungen, Lehrbuch der Pharmakologie und Toxikologie, Ernst Mutschler, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 6. Auflage von 1991, Kapt. 2.8.1 - Ovarien insbesondere Oestrogene, S. 322 bis S. 325 und Kap. 10.5.2 - Oestrogene und Antioestrogene, S. 677 bis 669

[D7] Reallexikon der Medizin und ihrer Grenzgebiete 5. Band, Urban & Schwarzenberg, 1973, S. O20, Östrogentherapie

[D8] J.Endocr., Vol. 102, 1984, 49-56, M. Axelson, J. Sjövall, B.E. Gustafsson, K.D.R. Setchell, Soya- a dietary source of the non-steroidal oestrogen equol in man and animals

[D9] Br. Med. J. 1990, 301: S. 905/906, G. Wilcox, M.L. Wahlqvist, H.G. Burger, G. Medley, Oestrogenic effects of plant foods in postmenopausal women

[D10] Acta Obstet. Gynaecol. Jpn., 1984, 36: 643-645, Mochimaru, F., Toyama M., Kanakura Y., Inde, S., Objective indicator for the assessment of postmenopausal hot flashes;– Übersetzung wird nachgeliefert

[D11] Technologie pflanzlicher Arzneizubereitungen, Paul Heinz List, Peter C. Schmidt, Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 1. Auflage von 1984, Kapt. 7- Die Weiterverarbeitung zum Fertigarzneimittel, S. 385 bis 389.

[D12] ASHP Statement on Unit Dose Drug Distribution, American Society of Hospital Pharmacists. Am. J. Hosp. Pharm., 1989, 46: 2346

[D13] Lehrbuch der pharmazeutischen Technologie, R. Voigt, VEB Verlag Volk und Gesundheit, Berlin, 5. Auflage, 1984, Kapt. 22 - Wäßrige Auszüge, Tinkturen, Extrakte, S. 439 bis 463 und Kapt. 24 - Stabilität und Stabilisierung S. 482 bis 483

[D14] Environmental quality and safety: EQS; Global Aspects of Chemistry, Toxicology, Stuttgart Thieme Verlag; Lindner H. R., Occurrence of Anabolic Agents in Plants and their Importance, S. 151-158

3. Gegenstand des Streitpatents

Das Streitpatent beinhaltet einen unabhängigen Anspruch 1 und weitere 10 auf diesen bezogene Unteransprüche (Ansprüche 2 bis 11).

Der unabhängige Anspruch 1 enthält dabei die folgenden Merkmale:

- Verwendung eines Isoflavon - Phytoöstrogen - Extraktes
- von Soja oder Klee
- zur Herstellung eines Medikamentes
- zur Verabreichung in Einzeldosierungsform
  - zur Behandlung des prämenstruellen Syndroms
  - zur Behandlung von menopausalen Symptomen oder
  - zur Behandlung von Prostatakrebs

Die nachgeordneten Ansprüche 2 bis 11 betreffen bevorzugte Ausführungsformen des Anspruchs 1

Anspruch 2 - das Medikament enthält zusätzlich ein nahrungsmittelgeeignetes Excipiens

Anspruch 3 - das Isoflavon-Phytoöstrogen wird aus Soja extrahiert

Anspruch 4 - das Isoflavon- Phytoöstrogen wird aus Sojahypokotylen extrahiert

Anspruch 5 - das Isoflavon-Phytoöstrogen wird aus Klee extrahiert

Anspruch 6 - der Isoflavon-Phytoöstrogen-Extrakt enthält einen oder mehrere Vertreter aus der Gruppe Genistein, Daidzein oder Glycoside deren Metabolite oder Derivate

Anspruch 7 - das Isoflavon-Phytoöstrogen enthält Genistein und/oder Biochanin A: Daidzein und/oder Formononetin in einem Verhältnis von ungefähr 1:2 bis 2:1 vorhanden sind

Anspruch 8 - die Isoflavon-Phytoöstrogene sind in einer Menge von ungefähr 20 mg bis 200 mg pro Dosiseinheit vorhanden

Anspruch 9 - die Verabreichung des Medikaments erfolgt wenigstens täglich über einen Zeitraumvon wenigstens einem Monat

Anspruch 10 - der Extrakt enthält Coumestane, Lignane und Flavone

Anspruch 11 - die Dosierungseinheitsform ist eine Tablette oder Kapsel

4. Mangelnde Offenbarung gemäß Art. 100 (b) i. V. m. Art. 83

Die Lösung der Aufgabe des Streitpatents – die Umwandlung des eigentlichen Wirkstoffes in ein Medikament in Einzeldosisform - wird nach Ansicht der Einsprechenden nicht ausreichend offenbart. Insbesondere die Umsetzung des technischen Merkmals, das Medikament in Einzeldosisform zur Verfügung zu stellen, wird von der Patentinhaberin unzureichend offenbart.

Bekannt ist aus dem Streitpatent, dass zunächst vorzugsweise ein flüssiger Extrakt aus der entsprechenden Pflanze vorliegt (S. 5 Absatz 0035), dieser stellt jedoch noch keine Einzeldosierungsform dar. Er wird durch Zugabe von Trägern oder Hilfsstoffen (S. 5, Zeile 29) zu einem Medikament formuliert. Die Beschreibung enthält jedoch keinerlei Angaben, welche Träger oder Hilfsstoffe eingesetzt werden können. Als Einzeldosierungsform werden Tabletten oder Kapseln bevorzugt (S. 7 Absatz 0058 und Anspruch 11).
Im Beispiel 1 wird die Herstellung des flüssigen Extraktes aus der Pflanze in den Absätzen 0069 bis 0072 beschrieben. Die Umwandlung in die Einzeldosierungsform – hier Tabletten - wird jedoch lediglich durch den Hinweis auf weitere zusätzliche Prozessschritte (S. 8, Zeile 50) wiedergegeben.

Gerade bei pflanzlichen Arzneiformen ist die Weiterverarbeitung zu den Endformulierungen mit mehr Schwierigkeiten als bei synthetischen Einzelstoffen verbunden - [D11] *Lehrbuch zur Technologie pflanzlicher Arzneizubereitungen, 1984, von Paul Heinz List und Peter C. Schmidt,* S. 385, rechte Spalte, 1. Absatz.

Je nachdem, ob der Extrakt zu Weichgelatinekapseln, Hartgelatinekapseln, Tabletten oder Dragees weiterverarbeitet wird, müssen unterschiedlichste Hilfsstoffe und Träger eingesetzt werden. Einen flüssigen Extrakt zu einer Einzeldosierungsform wie Kapseln und Tabletten umzuarbeiten, erfordert in der Regel eine Trocknung. Pflanzliche Trockenextrakte sind aufgrund schlechter Fließeigenschaften, einer ausgeprägten Hygroskopizität und eines tief liegenden Eutektischen Punktes meistens aber nicht direktverpressbar.
Für eine notwendige Granulierung oder die nachfolgende Tablettierung und eventuelle anschließende Dragierung sind daher meistens Hilfsstoffe erforderlich, die wiederum zu Unverträglichkeiten mit den Inhaltsstoffen des Extraktes führen können. Die Auswahl der Hilfsstoffe und Verfahrensparameter kann jedoch von entscheidender Bedeutung für die fertige Arzneiformulierung sein. Das Streitpatent schweigt zu einer genauen Lösung dieser Aufgabe.

Es ist somit nicht ausreichend offenbart wie die Patentinhaberin die Aufgabe löst, aus dem eigentlichen Wirkstoff, ein Medikament herzustellen, das in Einzeldosierungsform vorliegt.

5. Mangelnde erfinderische Tätigkeit gemäß Art. 56 EPÜ

Die Patentinhaberin selber gibt unter dem Kapitel „Background Art" einen umfangreichen Stand der Technik wieder, wobei entsprechende Literatur – hier in kursiver Schreibweise beispielhaft von der Einsprechenden angegeben - nicht benannt wurde:

a) es gibt pflanzliche Inhaltsstoffe mit östrogener Wirkung unter den Isoflavonen (S. 2, Absatz 0006)

[D1] *J. of Chromatography, 298 (1984) 175-182, J. Sachse, Quantitative Hochdruckflüssigkeitschromatographie von Isoflavonen in Rotklee – Absatz 1, Seite 175, insbesondere Zeile 10*

[D2] *Biochemical Journal, Vol.58, 1954, 283-287, D.H. Curnow, Oestrogenic Activity of Subterranean Clover-gesamter Artikel, beispielsweise S.283 Spalte 2 1. Absatz Fresh Clover Bioassay procedure i.V.m. S. 284 2. Spalte Tabelle 1 Partition of genistein and formononetin*

und mit anti-carcinogener Wirkung

[D3] *Cancer Research 48, 6257-6261, November 1988, John M. Cassady et al, Use of Mammalian Cell Culture...Inhibition of Metabolism by Biochanin A, an Isoflavone from Trifolium pratense (Herstellung ethanolischer Extrakt) - Abstract*

sowie unter den Lignanen (S.2, Absatz 0008) und Coumestanen (S.2, Absatz 0009).

[D4] *Food Additives and Contaminants, 1985, Vol.2, No.2, 73-106, Naturally occurring oestrogens in foods-A review, K.R. Price, G.R. Fenwick. – S. 85 Absatz Coumestans*

b) die Wirkmechanismen dieser Phyto-Östrogene (S. 3 Absätze 0012 bis 0014 und S. 4 Absatz 0026)

[D5] *Reproductive Toxicology, Vol.3, pp.81-89, 1989, Reproductive and General Metabolic Effects of Phytoestrogens in Mammals, Rami S. Kaldas et al. – Seite 83 Absatz Receptor activity and interaction, Seite 88 Phytoestrogens in Human Disease*

c) entsprechende Krankheiten, die abhängig vom Östrogenmetabolismus sind und mit der Gabe von Phyto-Östrogenen behandelt werden können (Seite 4 Absatz 0024).

[D5] *Reproductive Toxicology, Vol.3, pp.81-89, 1989, Reproductive and General Metabolic Effectsof Phytoestrogens in Mammals, Rami S. Kaldas et al. – Seite 88 Phytoestrogens in Human Disease*

[D6] *Arzneimittelwirkungen, Mutschler, 6. Auflage , 1991, Seite 324-325 übergreifender Absatz - Indikationen für Östrogene*

[D7] *Reallexikon der Medizin, 1973, Ansatz Östrogentherapie, S. O20*

d) das Vorkommen der Phyto-Östrogene in Kleesorten (S. 3, Absatz 0015 und S. 6 Absatz 0049)

[D1] *J. of Chromatography, 298 (1984) 175-182, J. Sachse, Quantitative Hochdruckflüssigkeitschromatographie von Isoflavonen in Rotklee – gesamter Artikel, insbesondere S.176, Ergebnisse und Diskussion Absätze 1 und 3*

[D2] *Biochemical Journal, Vol.58, 1954, 283-287, D.H. Curnow, Oestrogenic Activity of Subterranean Clover – gesamter Artikel, siehe Tabelle 2 oder Summary Punkt 2*

und in Sojabohnen (S.6 Absatz 0055),

[D4] *Food Additives and Contaminants, 1985, Vol.2, No.2, 73-106, Naturally occurring oestrogens in foods-A review, K.R. Price, G.R. Fenwick, Tabelle 1, Soya speziell Tabelle 3, S. 84 und Tabelle 5, S. 87.*

[D8] *J.Endocr., Vol. 102, 1984, 49-56, M. Axelson, J. Sjövall, B.E. Gustafsson, K.D.R. Setchell, Soya- a dietary source of the non-steroidal oestrogen equol in man and animals- abstract und S. 50 Absatz 2 – Isolation of daidzain from soya*

sowie

e) eine Studie, in der Frauen mit menopausalen Beschwerden nach einer Diät mit phyto-östrogenreichen Nahrungsmitteln wie Soya, Leinsamen oder Rotklee eine Erleichterung ihrer Beschwerden erfahren, die vergleichbar mit der Ersatztherapie mit synthetischen Östrogenen ist (S. 4 Absatz 0027).

[D9] *Br. Med. J. 1990, 301: S. 905/906, G. Wilcox, M.L. Wahlqvist, H.G. Burger, G. Medley, Oestrogenic effects of plant foods in postmenopausal women*

[D10] *Acta Obstet. Gynaecol. Jpn., 1984, 36: 643-645, Objective indicator for the assessment of postmenopausal hot flashes; Mochimaru, F., Toyama M., Kanakura Y., Inde, S..*

Nächstliegender Stand der Technik ist nach Ansicht der Einsprechenden die von der Patentinhaberin im Streitpatent auf Seite 4, Absatz 0027 Zeile 47-50 selber aufgeführte Studie, die belegt, dass Frauen im Klimakterium, die phyto-östrogenreiche Nahrungsmittel wie Soja,

Leinsamen oder Rotklee erhalten, eine Linderung ihrer menopausalen Beschwerden vergleichend einer synthetischen Östrogentherapie erfahren.
Im Unterschied zu dieser Studie setzt die Patentinhaberin phyto-östrogenreiche Extrakte von Soja oder Klee zur Herstellung eines Arzneimittels in Einzeldosisform ein, das zur gleichen Behandlung dienen soll.
Der Effekt dieses Unterschiedes ist, dass die eigentlichen Wirkstoffe in aufkonzentrierter Form vorliegen und gezielter appliziert werden können.

Die objektive Aufgabe besteht also darin, die phyto-östrogenen Wirkstoffe effizienter zur Behandlung von postmenopausalen Beschwerden zur Verfügung zu stellen.

Diese Aufgabe wird von der Patentinhaberin in zweistufiger Weise gelöst, einerseits damit, dass sie eine Verabreichung in aufkonzentrierter Form nämlich als Arzneimittel, das Extrakte aus Soja oder Klee enthält, vorschlägt, andererseits, dass sie eine Verabreichung in Einzeldosierungsform zur Verfügung stellt.
Dem Fachmann für medizinische Behandlungen oder Arzneimittel ist jedoch hinreichend bekannt, dass man pflanzliche Inhaltsstoffe aufkonzentrieren oder isolieren kann und entsprechend zu Arzneiformen weiterverarbeiten kann – siehe [D11] *Lehrbuch zur Technologie pflanzlicher Arzneizubereitungen, 1984, von Paul Heinz List und Peter C. Schmidt,* S.

Die Möglichkeit, dass sich phyto-östrogene Rotklee-Inhaltsstoffe auch aufkonzentrieren lassen, kann der Fachmann außerdem der [D1] *S. 175 Absatz 2,* unter *Extraktion* sowie in der [D2] *S. 284 linke Spalte* unter *Macro-method of estimation of genistein in clover* entnehmen, die die Herstellung eines Rotklee-Extraktes und die Aufkonzentrierung der Wirkstoffe beschreibt.

Der nacharbeitende Fachmann musste somit nicht erfinderisch tätig werden und die vermeintliche Erfindung wird durch den Stand der Technik nahegelegt.

Unabhängig von der Lösung, die Wirkstoffe in Form eines Arzneimittels aufzukonzentrieren und anzubieten, ist der Effekt, das Medikament „in Einzeldosierungsform" zu applizieren der, dass der Patient seine Arzneiform einfacher und sicherer einnehmen kann. Der Fachmann versteht unter

Einzeldosierungsformen Arzneiformen, die eine abgeteilte Einzeldosis enthalten, wie beispielsweise Kapseln oder Tabletten.
Objektive Aufgabe in Bezug auf das Merkmal „Einzeldosierungsform" wäre es somit, im Rahmen der effizienteren Anbietung die Verabreichung der phyto-östrogenen Wirkstoffe für den Patienten einfacher und sicherer zu gestalten.

Auch die Lösung dieser Aufgabe ist aus dem Stand der Technik nahegelegt. Die verbesserte und sichere Einnahme von Einzeldosierungsformen gehört zum Wissen eines Durchschnittsfachmannes und ist beispielsweise wiedergegeben in [D12] *ASHP Statement on Unit Dose Drug Distribution, American Society of Hospital Pharmacists. Am. J. Hosp. Pharm., 1989, 46: 2346.* Ein weiterer Vorteil gerade von Tabletten und Kapseln, nämlich die verbesserte Stabilität, ist ebenfalls in den einschlägigen Lehrbüchern zu Arneiformulierungen vermerkt ([D13] *Lehrbuch der pharmazeutischen Technologie, R. Voigt, VEB Verlag Volk und Gesundheit, Berlin, 5. Auflage, 1984*, S. 483, Z. 11 bis 18).

Keiner der Einzelschritte – das zur Verfügung stellen eines Arzneimittels oder die Verabreichung in Einzeldosierungsform – beruhen somit auf einer erfinderischen Tätigkeit. Auch die Kombination der Schritte wird durch den Stand der Technik in üblichen Lehrbüchern [D11] oder [D13] nahegelegt.

Vorausgesetzt die Patentinhaberin würde den nach Ansicht der Einsprechenden nicht erfinderischen Hauptanspruch durch Merkmale aus den Unteransprüchen ergänzen, wäre der neue Anspruch aus dem Stand der Technik immer noch bekannt:

So ist es üblich, dass ein Medikament - wie im **Anspruch 2** offenbart - zusätzlich einen nahrungsmittelgeeigneten Hilfsstoff oder Träger enthält – siehe Lehrbücher [D11] und [D13].

Dass das Isoflavon-Phytoöstrogen aus Soja (**Anspruch 3**) oder Sojahypokotylen (**Anspruch 4**) extrahiert wird, ergibt sich ebenfalls aus dem bekannten Stand der Technik [D4] Tabelle 5 S. 87.
Die Extraktion aus Rotklee (**Anspruch 5**) wurde bereits unter Anspruch 1 mit der [D1] S. 175 und [D2] auf S.284 abgehandelt.
Der Isoflavon-Phytoöstrogen-Extrakt soll laut **Anspruch 6** einen oder mehrere Vertreter aus der Gruppe Genistein, Daidzein oder Glycoside deren Metabolite oder Derivate enthalten. Auch dieses Merkmal ist gemäß [D4] auf S. 75 letzte zwei Zeilen; [D1] auf S.177 und für Genistein aus der [D2] nahegelegt.
Anspruch 7 beansprucht, dass das Isoflavon-Phytoöstrogen Genistein und/oder Biochanin A: Daidzein und/oder Formononetin in einem Verhältnis von ungefähr 1:2 bis 2:1 enthält. Da die Verteilung der Inhaltsstoffen dem Vorkommen in der Pflanze entspricht ist auch dieses Merkmal nicht erfinderisch. Als Beispiel soll die [D14] dienen *Environmental quality and safety: EQS; Global Aspects of Chemistry, Toxicology, Stuttgart Thieme Verlag; Lindner H. R., Occurrence of Anabolic Agents in Plants and their Importance, S. 151-158.* Auf S. 154 ist die Zusammensetzung der Isoflavone aus Sojabohnen angegeben. Nimmt man die Menge an Genistein (18,6) zu Daidzin (30.0) oder Genistein (18,6) zu Daidzin (30,0) und Formononentin (4,3) so liegen diese Mengenverhältnisse in dem beanspruchten Bereich.
Eine wie in **Anspruch 8** offenbarte Dosis an Isoflavon-Phytoöstrogenen ist von dem jeweiligen Extraktes und der eingesetzten Applikationsform abhängig und kann im Einzelnen durch Versuche ermittelt werden. Sie macht die Herstellung eines Medikamentes oder eine Anwendung nicht neu oder erfinderisch, wenn die üblichen zu dieser Indikation eingesetzten Dosen an Östrogenen bekannt sind und dieser Dosis entsprechen.
Das gleiche gilt für die Verabreichung eines Medikaments - **Anspruch 9**. Sie erfolgt auch bei der synthetischen Östrogentherapie wenigstens täglich und über einen Zeitraum von wenigstens einem Monat.

Auch **Anspruch 10** macht den Gegenstand des Patentes nicht erfinderisch, so ist beispielsweise bekannt, dass bestimmte Kleesorten Coumestane enthalten [D4] – S.85 Absatz Coumestans in Ladino Kleesorten.
Die in **Anspruch 11** beanspruchte Dosierungseinheitsform einer Tablette oder Kapsel wurde schon ausführlich unter Anspruch 1 abgehandelt.

Somit ist nach Ansicht der Einsprechenden die Anforderung an die erfinderische Tätigkeit für den unabhängigen Anspruch 1 nicht erfüllt und auch die beschreibenden Merkmale aus den Unteransprüchen 2 bis 11 eignen sich nicht zur Erklärung des erfinderischen Schrittes.

6. Zusammenfassung

Zusammenfassend ist die Einsprechende somit der Ansicht, dass das Streitpatent die Anforderungen an die erfinderische Tätigkeit gemäß Artikel 56 EPÜ nicht erfüllt. Des Weiteren ist gemäß Artikel 100 b) EPÜ die Erfindung nicht so deutlich und vollständig offenbart, dass ein Fachmann sie ausführen kann. Sie beantragt daher den Widerruf des Patentes in vollem Umfang und allen benannten Vertragsstaaten.

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Dr. Jürgen Reinhardt Dr. Heike Kublik
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Anlage

Zahlung von Gebühren und Auslagen

An das
Europäische Patentamt
Direktion Kassen-
und Rechnungswesen

Name des Einzahlers
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Aktenzeichen der Patentanmeldung/des Patents (für jedes Aktenzeichen ein Formblatt)
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Cognis Deutschland GmbH & Co. KG

Düsseldorf, den 13.06.2005

U. Wolter

Dr. Kublik

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DA

Note

Quantitative Hochdruckflüssigchromatographie von Isoflavonen in Rotklee (*Trifolium pratense* L.)

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Die quantitative Bestimmung von Isoflavonen ist wegen der östrogenen, antoxidierenden, fungiziden und antihämolytischen Eigenschaften[1–3] dieser Substanzen von Bedeutung. Bisher konnten diese Stoffe nach der klassischen säulen- und dünnschichtchromatographischen Reinigung photometrisch erfasst werden[4]. Die Einführung der Hochdruckflüssigchromatographie (HPLC) ermöglicht es, das erste arbeits- und zeitaufwendige Verfahren durch ein rascheres zu ersetzen, worüber bereits einige Publikationen vorliegen. Die Untersuchungen betreffen Reinsubstanzen[5] sowie die isoflavonhaltigen Pflanzen *Glyzine soja* L.[6–11], *Trifolium subterraneum* L.[12,13] und *Cicer arietinum* L.[14], die für die menschliche oder tierische Ernährung angebaut werden. Ein Teil der östrogenen Isoflavone wurden sowohl als Glycoside als auch als Aglycone bestimmt. Diese Isoflavone kommen fast nur in den Leguminosen vor, ihre qualitative und quantitative Zusammensetzung ist jedoch in jeder Pflanzenart verschieden, was ein Vergleich zwischen den naheverwandten Arten Rot- und Weissklee zeigt[4]. Ausserdem erfordert wegen des unterschiedlichen komplexen Gehaltes an Begleitstoffen jedes Untersuchungsobjekt eine andere Bestimmungsmethode, so dass vorhandene Verfahren nicht ohne weiteres übernommen werden können. Dies bezieht sich sowohl auf die Extraktion als auch auf die Auftrenntechnik mit HPLC.

Das Ziel dieser Arbeit war es, für die quantitative Bestimmung der fünf Isoflavonaglykone Biochanin A, Formononetin, Pratensein, Genistein und Daidzein in Rotklee mit Hilfe der HPLC eine Methode zu entwickeln.

EXPERIMENTELLER TEIL

Extraktion

Man lässt 1 g gefriergetrocknetes, feingemahlenes Material (dreizehn Rotklee-Zuchtstämme, Ernte 1983, 2. Schnitt) mit 30 ml Wasser 1 h quellen. Nach Zugabe von 100 ml Äthanol (95%) den Ansatz 1 h rühren oder über Nacht stehen lassen. Den festen Anteil abnutschen, mit Äthanol gut nachwaschen und das Filtrat bei 50°C am Rotationsverdampfer eindampfen. Den Rückstand mit 15 ml 2 *N* Salzsäure 1 Stunde im siedenden Wasserbad hydrolysieren. Nach dem Neutralisieren mit konz. Natronlauge auf pH 5–6 das Hydrolysat mit heissem Äthanol (95%) quantitativ in einen 50 ml Messkolben spülen. Für die Identitäts- und Reinheitsprüfung der Peaks einen Extrakt aus 3 g Klee/100 ml nach dem gleichen Verfahren herstellen.

0021-9673/84/$03.00

HPLC

Gerät: Perkin-Elmer Liquid Chromatograph Series 3B, Rheodyne Probenaufgeber 7125 mit 20 µl Schleife. Detektor: Perkin Elmer Spectrometric Detector LC 75 mit Autocontrol. Schreiber: W + W Recorder 1100. Integrator: Spectra-Physics Autolab System I.

(a) HPLC von Reinsubstanzen: Hibar-Fertigsäule Merck 250 × 4 mm, LiChrosorb RP-18, 10 µm. Eluent: Acetonitril (Mallinckrodt Chromar)–bidestilliertes Wasser. Gradient: linear, 15 min, 30 bis 80% Acetonitril. Fliessgeschwindigkeit: 1.8 ml/min Papiervorschub: 1 cm/min. Detektion: 260 nm. Schreibervollausschlag bei einer Extinktion von 0.64. Isoflavonkonzentration: 1 µg/20 µl.

(b) HPLC der Klee-Extrakte: Hibar-Fertigsäule Merck 250 × 4 mm, LiChrosorb RP-18, 5 µm. Eluent: Acetonitril (Mallinckrodt Chromar)–bidestilliertes Wasser mit einem Zusatz von 0.5% (v/v) Phosphorsäure 85% (Merck p.a.) bzw. 0.5% (v/v) Ameisensäure 98–100% (Merck p.a.). Gradient: 10 min 0.1–20% Acetonitril linear, 42 min 20–33% Acetonitril linear, 10 min 33–37 Acetonitril konkav, 20 min, 40% Acetonitril isokratisch bei Raumtemperatur. Fliessgeschwindigkeit: 0.9 ml/min. Detektion: 260 nm, Schreibervollausschlag bei einer Extinktion von 0.16. Papiervorschub: 0.1 cm/min. Integration: Externer Standard, Herkunft der Isoflavone[4]. Standardlösung: Je 5 mg der Isoflavone in 50 ml Äthanol lösen. Etwa 60 µl dieser Standardlösung bzw. des Extraktes im 50 ml Messkolben injizieren.

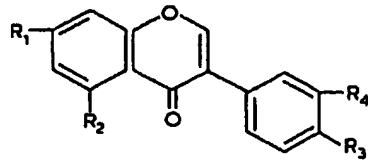
Das angefallene Acetonitril auf folgende Weise vernichten: 2 l des Gemisches in einem 3-l Rundkolben mit 400 g techn. Natriumhydroxid in drei Portionen versetzen. Mit aufgesetztem Kühler in einem Abzug gut schütteln, bis zur nächsten Zugabe abkühlen lassen. Das Acetonitril trennt sich bei der ersten Portion Natriumhydroxid von der wässrigen Phase. Nach der letzten Zugabe einige Siedesteine beifügen, das Gemisch in einer Heizkalotte bis zum Sieden der oberen Phase unter guter Kühlung im Abzug erhitzen bis keine zwei Phasen mehr sichtbar sind (etwa 3–4 h). Nach dem Abkühlen kristallisiert der Kolbeninhalt in langen Kristallnadeln fast ohne Flüssigkeitsrest aus.

ERGEBNISSE UND DISKUSSION

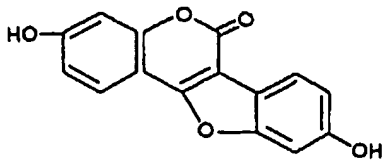
Die gewonnenen Ergebnisse über den Gehalt an Isoflavonen in Rotklee-Zuchtstämmen, die verschiedenen morphologischen Gruppen angehören, zeigen Formononetin und Biochanin A als Hauptkomponenten (Tabelle I). Genistein und Daidzein mit drei bzw. zwei Hydroxylgruppen am Molekül (Fig. 1) können als Vorläufer von Biochanin A und Formononetin betrachtet werden, die in der Pflanze durch Methylierung der Hydroxylgruppe an C-4′ zu stabileren sekundären Stoffwechselprodukten überführt werden.

Von den dreizehn untersuchten Genotypen besitzen die Zuchtstämme vier den niedrigsten (398 mg) und acht den höchsten Gehalt (739 mg) an Biochanin A. Für Formononetin sind es die entsprechenden Stämme 11 und 12 mit rund 780 und 980 mg dieses Isoflavons.

Wie Fig. 2 zeigt, lassen sich die fünf Isoflavone einschliesslich des Cumarinderivates Cumöstrol, das ebenfalls in Luzerne und Kleearten gefunden wurde und östrogene Eigenschaften besitzt[15,16], innerhalb von 11 min trennen. Da bekanntlich polare Substanzen vor weniger polaren von einer Umkehrphasensäule eluiert werden,

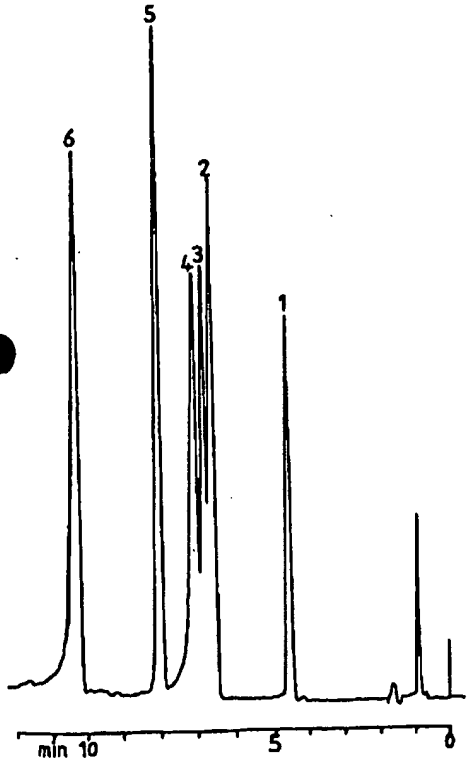


	R_1	R_2	R_3	R_4
Daidzein	OH	H	OH	H
Genistein	OH	OH	OH	H
Pratensein	OH	OH	OCH_3	OH
Formononetin	OH	H	OCH_3	H
Biochanin A	OH	OH	OCH_3	H



Cumöstrol

Fig. 1. Strukturformel von einigen Isoflavonen und Cumöstrol.



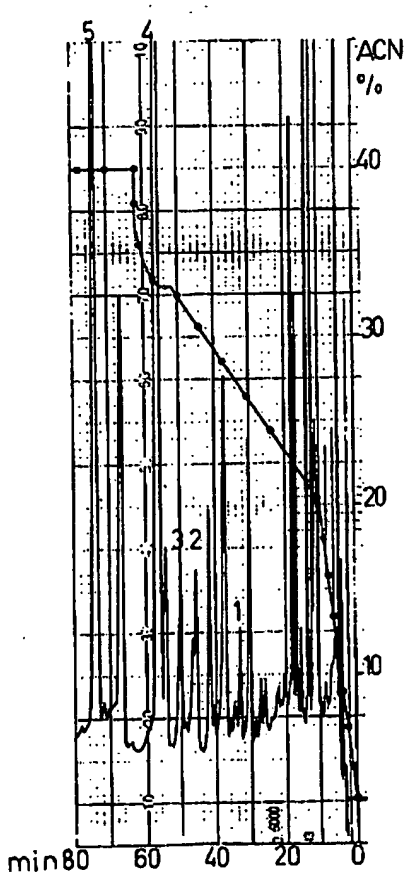


Fig. 2. HPLC-Chromatogramm von fünf Isoflavonen und Cumöstrol. 1 = Daidzein, 2 = Genistein, 3 = Cumöstrol, 4 = Pratensein, 5 = Formononetin, 6 = Biochanin A. Chromatographische Bedingungen siehe experimentellen Teil.

Fig. 3. HPLC-Chromatogramm eines Rotklee-Extraktes. 1 = Daidzein, 2 = Genistein, 3 = Pratensein, 4 = Formononetin, 5 = Biochanin A. Chromatographische Bedingungen siehe experimentellen Teil.

TABELLE I

ISOFLAVONGEHALT EINIGER ROTKLEEZUCHTSTÄMME (mg/100 g TROCKENSUBSTANZ)

Die Einzelwerte wurden durch Doppelbestimmungen ermittelt.

Stamm-Nr.	*Biochanin A*			*Formononetin*			*Pratensein*			*Genistein*			*Daidzein*		
	x	$\bar{x}$	d^*	x	$\bar{x}$	d	x	$\bar{x}$	d	x	$\bar{x}$	d	x	$\bar{x}$	d
1	519	513	1.2	848	832	1.9	53	56	4.3	82	83	1.2	18	17	5.6
	507			815			58			84			16		
2	457	443	3.2	865	837	3.2	82	82	0	66	67	0.7	13	14	3.6
	428			809			82			67			14		
3	511	506	1.0	830	850	2.3	57	57	0	77	68	11.7	12	13	3.8
	501			870			57			61			13		
4	398	398	0	896	895	0.2	56	61	6.9	50	57	10.9	12	13	3.8
	398			893			65			64			13		
5	492	493	0.1	887	907	2.1	66	64	3.0	70	72	2.1	4	6	21.4
	493			926			62			73			7		
6	606	618	1.9	939	960	2.1	55	55	0	24	25	2.0	3	3	0
	630			980			55			25			3		
7	450	450	0.1	857	820	4.4	51	50	2.0	54	54	0.9	7	6	14.3
	449			781			49			53			5		
8	764	739	3.3	849	832	2.1	76	68	11.2	44	48	6.9	4	4	0
	713			814			59			51			4		
9	526	529	0.6	843	841	0.3	65	62	5.4	59	62	3.9	9	9	5.6
	532			838			58			64			8		
10	504	521	3.2	753	765	1.6	78	80	2.4	57	85	0.9	7	6	21.4
	538			777			82			58			4		
11	495	510	2.9	749	779	3.7	51	49	3.9	64	57	10.9	4	5	10.4
	525			809			47			50			5		
12	515	518	0.6	978	979	0.1	52	53	1.0	44	45	1.1	7	7	0
	521			980			53			45			7		
13	466	489	4.4	983	954	3.0	50	51	1.0	42	41	2.4	6	6	0
	511			925			51			40			6		
			$\bar{d} = 1.7$			$\bar{d} = 2.1$			$\bar{d} = 3.1$			$\bar{d} = 4.3$			$\bar{d} = 6.9$

* d = Prozentuale Abweichung der Einzelwerte x vom Mittelwert $\bar{x}$. $\bar{d}$ = Mittlere prozentuale Abweichung.

ergibt sich für die sechs Substanzen die Reihenfolge: Daidzein, Genistein, Cumöstrol, Pratensein, Formononetin und Biochanin A. Obgleich Daidzein eine Hydroxylgruppe weniger als Genistein besitzt (Fig. 1), wird es vor letzteren eluiert. Der Wasserstoff der Hydroxylgruppe an C-5 ist durch Van der Waalssche Kräfte an die Keto gruppe an C-4 gebunden, wodurch Genistein lipophilere Eigenschaften erhält. Das gleiche gilt für das Isoflavonpaar Formononetin und Biochanin A. Eine Methoxylgruppe statt einer Hydroxylgruppe an C-4′ macht die Verbindung unpolarer, infolgedessen werden Daidzein, Genistein und Cumöstrol vor Pratensein, Formononetin und Biochanin A eluiert. Durch eine dritte Hydroxylgruppe an C-3′ wird Pratensein vor Formononetin und Biochanin A, aber wegen der Methoxygruppe an C-4′ nach Genistein von der Säule abgelöst. Cumöstrol ist mit zwei Hydroxylgruppen an C-7 und C-4′, einer Sauerstoffbrücke zwischen C-4 und C-6′ und der Ketogruppe an C-2 weniger polar als Genistein.

Der aus 1 g Probenmaterial erhaltene Extrakt enthält noch so viele bei 260 nm absorbierende Substanzen, dass die Bestimmung der fünf Isoflavone, deren Absorptionsmaxima bei 250 und 263 nm liegen, nicht mehr in kurzer Zeit erfolgen kann, wenn möglichst nicht verunreinigte Peaks erhalten werden sollen. Ausserdem war es vorteilhaft, die Ionisation der Isoflavone durch Säurezusatz im Eluenten B zu unterdrücken und somit schärfere Peaks zu erzielen. Dafür boten sich sowohl Phosphorsäure als auch Ameisensäure[5] in einer Konzentration von 0.5% (v/v) an. Höhere

TABELLE II

IDENTIFIKATION UND REINHEITSPRÜFUNG DER ISOFLAVONPEAKS

Messung jedes Isoflavonpeaks an drei Punkten.

Isoflavone	*Extinktionsquotient "absorbance ratio"*						*Extinktionsbereich der Isoflavone aus dem Extrakt*
	240/257 nm		*257/230 nm*		*240/310 nm*		
	Standard	*Extrakt*	*Standard*	*Extrakt*	*Standard*	*Extrakt*	
Daidzein	1.00	1.03	2.43	2.58	2.46	2.67	0.011–0.032
	1.01	1.03	2.45	2.58	2.47	2.67	
	1.01	1.04	2.48	2.18	2.50	2.27	
Genistein	0.45	0.38	5.98	8.67	2.78	3.33	0.006–0.025
	0.46	0.48	6.03	4.16	2.81	2.00	
	0.46	0.57	6.11	3.50	2.83	2.00	
Pratensein	0.50	0.71	4.08	3.17	2.11	2.25	0.012–0.055
	0.52	0.65	4.09	3.43	2.13	2.25	
	0.51	0.68	4.08	3.15	2.11	2.15	
Formononetin	0.95	0.96	2.57	2.58	2.45	2.49	0.199–1.036
	0.96	0.96	2.57	2.58	2.47	2.49	
	0.96	0.96	2.57	2.60	2.48	2.50	
Biochanin A	0.46	0.43	6.11	6.00	2.83	2.94	0.016–0.297
	0.47	0.45	6.19	6.18	2.89	2.81	
	0.48	0.45	6.17	6.30	2.96	2.84	

TABELLE III

REINHEITSPRÜFUNG DER ISOFLAVONPEAKS MIT DEM "ABSORBANCE INDEX" Q*

Standard			*Wellenlänge (nm)*									*Diskriminator***
			210	*220*	*230*	*240*	*250*	*260*	*270*	*280*	*290*	
Daidzein	Extinktion · 10^{-3}	*A*	281	199	228	296	334	284	222	150	122	
		B	202	143	161	211	238	202	156	106	87	
	Q		1.39	1.39	1.42	1.40	1.40	1.41	1.42	1.42	1.40	1.02
Genistein	Extinktion · 10^{-3}	*A*	518	368	268	330	527	779	549	305	225	
		B	552	349	255	313	500	735	525	291	215	
	Q		1.05	1.05	1.05	1.05	1.05	1.06	1.06	1.05	1.05	1.01
Pratensein	Extinktion · 10^{-3}	*A*	264	204	136	117	170	254	206	121	103	
		B	237	181	122	106	153	225	185	107	91	
	Q		1.11	1.13	1.11	1.10	1.11	1.13	1.11	1.13	1.13	1.03
Formononetin	Extinktion · 10^{-3}	*A*	203	148	167	221	251	229	177	115	88	
		B	151	109	124	163	184	169	132	86	65	
	Q		1.34	1.36	1.35	1.36	1.36	1.36	1.34	1.34	1.35	1.01
Biochanin A	Extinktion · 10^{-3}	*A*	271	185	129	152	238	354	277	157	111	
		B	183	125	88	103	160	243	189	107	77	
	Q		1.48	1.48	1.47	1.48	1.49	1.46	1.47	1.47	1.44	1.03
Aus Rotklee												
Formononetin	Extinktion · 10^{-3}	*A*	545	398	449	572	642	577	454	301	240	
		B	275	203	231	294	329	293	230	153	124	
	Q		1.98	1.96	1.94	1.95	1.95	1.97	1.97	1.97	1.94	1.02
Biochanin A	Extinktion · 10^{-3}	*A*	116	77	54	62	97	141	111	64	46	
		B	88	59	40	47	71	106	82	47	34	
	Q		1.32	1.31	1.35	1.32	1.37	1.33	1.35	1.36	1.35	1.05

* $Q = A/B$; $A + B$ = Extinktion an zwei verschiedenen Punkten eines Peaks.
** Diskriminator = höchster Q-Wert/tiefsten Q-Wert.

Konzentrationen brachten keine weiteren Vorteile. Die bessere Auftrennung wird mit Ameisensäure erreicht, die einerseits Ionendepression ermöglicht, anderseits auch als Lösungsmittel für die Isoflavone wirkt. Nachteilig ist die Eigenabsorption der Ameisensäure unter 250 nm, die die Aufnahme von Absorptionsspektren und eine Reinheitsprüfung der Isoflavonpeaks durch "absorbance ratio" und den "absorbance index" (siehe unten) ausschliesst. Fig. 3 zeigt ein Chromatogramm eines Rotklee-Extraktes. Peaks 1–5 wurden mit Vergleichssubstanzen und an Hand ihrer Absorptionsspektren, die direkt während der unterbrochenen Elution mit Hilfe des LC-75-Autocontrol-Detektors erhalten wurden, identifiziert. Allerdings sagen die Absorptionsspektren über eventuelle Verunreinigungen eines Peaks wenig aus. Dies wurde aus dem Quotienten zweier Extinktionen bei verschiedenen Wellenlängen an einem Peak ermittelt und ergibt die "absorbance ratio" (Tabelle II)[17]. Die Extinktionsmessung erfolgte bei 240, 257 und 310 nm an der auf- und absteigenden Seite des Peaks sowie auf seinem Scheitelpunkt. Der Peak ist dann nicht verunreinigt, wenn die erhaltenen Quotienten an allen drei Messpunkten wenig von einander abweichen und den Werten der Standardsubstanzen entsprechen. Dies trifft für Biochanin A und Formononetin eindeutig, für Daidzein annähernd und für Genistein und Pratensein nicht mehr zu. Allerdings muss erwähnt werden, dass konstante Extinktionsquotienten nur dann zu erhalten sind, wenn die Extinktionen nicht unter 0.05 liegen, da sonst geringe Messabweichungen bereits wesentliche Veränderungen der Quotienten bedingen. Diese Methode erlaubt es gleichzeitig zu erkennen, ob die Verunreinigung vor oder nach dem interessierenden Peak eluiert wird. Ausserdem lässt sich ermitteln, ob zwei schlecht getrennte Peaks auf einen Riss in der Säule zurückzuführen sind oder tatsächlich zwei verschiedene Substanzen bedeuten. Ein Urteil über die Trennleistung einer Säule nach längerem Gebrauch ist durch diese Technik ebenfalls möglich.

Ein weiterer Test auf verunreinigte Peaks stellt der "absorbance index"[18] dar, bei dem die Extinktionen eines Peaks an zwei verschiedenen Punkten über das Absorptionsspektrum der entsprechenden Substanz z.B. aller 10 nm von 210 bis 290 nm gemessen werden. Der Quotient aus den beiden Werten sollte bei jeder Wellenlänge gleich sein bzw. nicht mehr als 2% abweichen. Das wird erreicht, wenn die Extinktionen hoch sind, diese sich bei derselben Wellenlänge durch Änderung der Konzentrationen in der Messzelle wesentlich unterscheiden, so dass daraus ein hoher Quotient resultiert (Tabelle III). Mit Eichsubstanzen lässt sich dies ohne Mühe erzielen, im Rotklee-Extrakt liegen jedoch nur Formononetin und Biochanin A in ausreichender Konzentration vor. Hingegen sind die Mengen an Daidzein, Genistein und Pratensein eindeutig zu gering, so dass das Verhältnis des Signals zum Rauschen sehr ungünstig wird. Eine starke Konzentrierung des Extraktes würde die Trennleistung der verwendeten Säule durch den grossen Anteil an Begleitstoffen übersteigen. Eine verlustlose Vorreinigung der Extrakte mit einer Sep-Pak C_{18} Cartridge von Waters Associates ermöglicht zwar die Abtrennung der vor Daidzein eluierbaren Stoffe, vereinfacht aber das eigentliche Trennproblem nicht.

Da sich Rotklee-Extrakte in ihrer chemischen Zusammensetzung und in ihren physikochemischen Eigenschaften unterscheiden können, was sich schon oft beim Extrahieren und Filtrieren bemerkbar macht, kann die Auftrennung der Isoflavone durch HPLC in ihrer Qualität schwanken. Wegen der zahlreichen unbekannten und womöglich chemisch sehr ähnlichen Begleitstoffe muss damit gerechnet werden, dass eventuelle Verunreinigungen gleiche Retentionszeiten wie die interessierenden Substanzen besitzen und infolgedessen nicht von einander trennbar sind.

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Oestrogenic Activity of Subterranean Clover

2. THE ISOLATION OF GENISTEIN FROM SUBTERRANEAN CLOVER AND METHODS OF QUANTITATIVE ESTIMATION

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The isolation of small amounts of genistein (5:7:4'-trihydroxy-*iso*flavone) from subterranean clover (*Trifolium subterraneum* var. *Dwalganup*) and the demonstration of its weakly oestrogenic properties (Bradbury & White, 1951, and Biggers & Curnow, 1954), together with the difficulties in the synthesis of this compound, made it important that a more efficient method be found for the isolation of this *iso*flavone from the clover. Also, the amount of genistein isolated by Bradbury & White (2 mg./100 g. of fresh clover) and its low oestrogenic activity (Biggers & Curnow, 1954) were not nearly sufficient to account for the activity of the fresh plant (Robinson, 1949) and left considerable doubt as to whether this compound was responsible for the oestrogenic activity of the clover.

The present study has shown, however, that the clover contains much more genistein than found by these workers. The amount now found indicates that genistein could indeed be responsible for the activity of the plant and therefore for the infertility in sheep grazing on subterranean-clover pastures. This finding called for rapid and efficient methods of estimation of this compound in the plant. For the determination of the genistein content of the clover at various stages of growth, under various nutritional conditions, in different seasons and in various strains, an accurate method of estimation was required, whereas for the purposes of the plant geneticist a rapid method was desired for use with small amounts of the material (parts of a single plant), in which great accuracy is a secondary consideration. Methods fulfilling these two sets of requirements have been developed and are presented here.

EXPERIMENTAL

Materials

'Chloroplast fraction'. The 'chloroplast fraction' was prepared as described by Bradbury & White (1951) from the *Dwalganup* strain of subterranean clover. From 4020 kg. of fresh plant 15 kg. of 'chloroplast' was produced.

Fresh clover. Clover was cut and weighed in the field and placed immediately in ethanol.

Silica gel. Davison Chemical Corporation '200 mesh' silica gel was used.

Bioassay procedure. The methods described in the previous paper (Biggers & Curnow, 1954) were used for the assay of the oestrogenic activity of extracts.

Isolation of genistein from 'chloroplast'

Extraction. An amount of 100 g. of 'chloroplast' was extracted with 1 l. ethanol for 30 min. while being stirred under reflux. The residue was extracted with a further 500 ml. ethanol, under reflux for 15 min. The combined extract contained 7·2 g. of solid. Extraction of the chloroplast residue with ethanol and with ethanolic NaOH failed to yield any further oestrogenic material.

Purification. The combined ethanol extracts were evaporated *in vacuo* to a volume of 600 ml. Water was added to bring the volume to 1000 ml. and the mixture extracted 4 times with 200 ml. benzene. The benzene extracts were washed in countercurrent fashion 4 times with 200 ml. 60% (v/v) ethanol. This treatment removed from the aqueous ethanol all the chlorophyll and fats, and left a clear pale-yellow solution. The dark-green benzene phase contained 2·78 g. of solid which showed no oestrogenic activity on injection in ovariectomized mice either before or after saponification.

The aqueous ethanol phase was evaporated *in vacuo* to a volume of 200 ml. and extracted 4 times with 200 ml. of ether. The aqueous phase after concentration showed no oestrogenic activity. The ether phase was extracted 4 times with 20 ml. saturated aqueous $NaHCO_3$ and then twice with 20 ml. water. (The dark brown aqueous extract was acidified with H_2SO_4 and extraction with ether yielded 0·74 g. of material with no oestrogenic activity.) The ether phase contained 3·50 g. of light yellow solid with oestrogenic activity equal to that of approx. 6 μg. oestradiol. Chromatography of 250 mg. of this material on 50 g. silica gel, by using light petroleum–ether mixtures and determining the ultraviolet absorption curves of the eluates to locate the genistein fractions, as in the macro-method below, yielded 30 mg. of genistein, which after recrystallization from aqueous ethanol gave m.p. 300–301° (decomp.) (corr.) not depressed by a synthetic sample. Thus 100 g. of 'chloroplast' yielded 420 mg. of genistein. Related to the original fresh clover this is equivalent to about 2 mg./100 g. (12 mg./100 g. dry matter), which is the figure given by Bradbury & White (1951).

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Isolation of genistein from fresh clover

From 100 g. fresh clover (16 g. dry matter), treated precisely as for the 'chloroplast', 59 mg. pure genistein were isolated. This is equivalent to 369 mg. genistein/100 g. dry matter of clover.

Macro-method of estimation of genistein in clover

Sampling. Samples of 30 g. fresh clover were taken from the field and placed immediately in 200 ml. ethanol. A separate sample was taken for drying and estimation of dry-matter content.

Extraction. The fresh clover was refluxed with the 200 ml. ethanol for 15 min., cooled and filtered. The residue was macerated with a further 300 ml. ethanol in a Townson and Mercer (Croydon, Surrey) top-drive macerator and refluxed for 30 min., cooled and filtered. The macerated residue was washed with 50 ml. ethanol and all the ethanol extracts were combined.

Extraction of the residue, either with ethanol or with ethanolic NaOH, failed to yield any extract with oestrogenic activity.

Aqueous ethanol–benzene partition. The combined ethanol extracts were evaporated *in vacuo* to 150 ml. and water was added to bring the volume to 250 ml. The resulting 60% ethanol solution was extracted with three 50 ml. portions of benzene, the separating funnel being gently rotated to avoid the formation of troublesome emulsions. The three benzene extracts were kept apart and extracted in counter-current fashion with five 50 ml. portions of 60% ethanol.

The benzene extract was evaporated to dryness, hydrolysed under reflux with 100 ml. ethanolic N-NaOH and the 'phenol' fraction isolated in the usual way. Injection in ovariectomized mice showed that it contained less than 5% of the total oestrogenic activity of the original extract.

Thus the fats and chlorophylls were retained in the benzene phase whereas the clear, faintly yellow aqueous-ethanol phase contained more than 95% of the oestrogenic material.

The partition coefficient of genistein between benzene and aqueous ethanol was estimated in the following manner. Samples of 0·5 mg. genistein were shaken with 50 ml. aqueous ethanol (50, 60 and 70%, v/v) and 50 ml. benzene and the phases allowed to separate. The temperature was 19°. The aqueous ethanol phases were evaporated to dryness *in vacuo* and the residues dissolved in 50 ml. ethanol. The extinction at 262·5 mμ. was determined with a Beckman spectrophotometer. Similar determinations were made for formononetin (7-hydroxy-4′-methoxy-*iso*flavone) by using the extinction at 249 mμ. The results are given in Table 1.

The absorption curves of genistein and formononetin in pure ethanol are shown in Fig. 1. Over the range 0·1 mg./100 ml. the optical density at 262·5 mμ. of genistein solutions was found to be directly proportional to the concentration.

With the partition ratio of 1·39 for genistein between 60% ethanol and benzene, the theoretical recovery of genistein in the purification procedure is 97·9%. For formononetin the theoretical recovery is 59·3%. Very little formononetin, however, is found in the purified clover extracts. Formononetin is relatively insoluble in ethanol (175 mg./100 ml. at 22°), whereas genistein is readily soluble.

Separation of phenols. The aqueous ethanol solution was evaporated *in vacuo* to a volume of 100 ml., saturated with NaCl and extracted with four 100 ml. portions of ether. The combined ether solution was then extracted with six 25 ml. portions of 0·1 N-NaOH. CO_2 was passed into the combined NaOH extracts until they were saturated, and the phenols were extracted with four 100 ml. portions of ether. The combined ether solution was washed with three 20 ml. portions of water, and evaporated to dryness to yield the phenols.

Chromatography. An amount of silica gel 200 times the weight of the phenols was transferred with stirring into a water-jacketed column 15 mm. in diameter filled with light petroleum (40–60°) and the phenols were chromatographed on this column with 50 ml. portions of dry light petroleum–ether mixtures as eluting solvents, running from pure light petroleum to 10, 20, 30...100% (v/v) ether. Finally acetone and then methanol were run to elute the column completely. The pressure at the top of the column was increased by nitrogen at 50–150 mm. Hg. From 20 to 30 fractions were obtained in this way. These fractions were weighed and the recoveries from a number of columns varied from 100 to 110%, the excess being due presumably to some material eluted from the silica gel.

Table 1. *Partition of genistein and formononetin between aqueous ethanol and benzene*

The partition ratio (concentration in aqueous ethanol/conen. in benzene) was determined using 50 ml. volumes of the two phases and 0·5 mg. of the substance investigated. The concentrations in the extracts were determined by spectrophotometry. Genistein, $E^{1\%}_{1cm}$ 262·5 mμ. (max.) = 1400. Formononetin, $E^{1\%}_{1cm}$ 249 mμ. (max.) = 1000.

	Aqueous phase		
Substance	50% ethanol (v/v)	60% ethanol (v/v)	70% ethanol (v/v)
Genistein	0·975	1·39	2·37
Formononetin	0·185	0·361	0·645

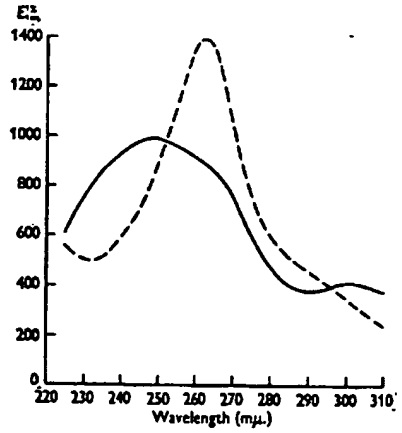

Fig. 1. Absorption curves of genistein (– – –) and formononetin (——) in ethanol solution.

Spectrophotometry. range 220–300 mμ. solution in ethanol fractions is shown in

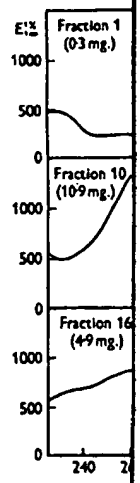

Fig. 2. Ultraviolet fractions of Fractions 10, 1 and 21 do not.

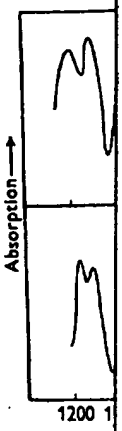

Fig. 3. Infrared and (*b*) chroma Bands due to are dotted. (C with sodium paraffin oil.)

Spectrophotometry. The absorption spectrum, over the range 220–300 mμ., was observed for each fraction in solution in ethanol. A typical series of curves for the fractions is shown in Fig. 2.

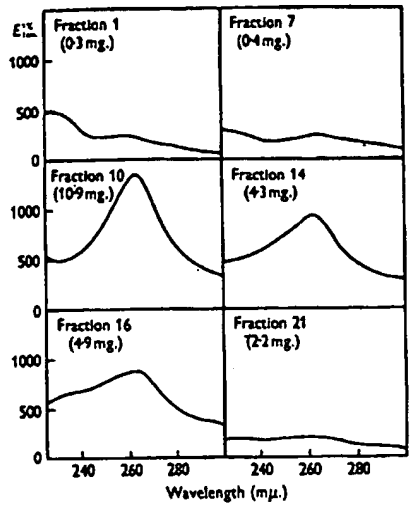

Fig. 2. Ultraviolet absorption of a series of chromatograph fractions of subterranean-clover phenolic material. Fractions 10, 14 and 16 contain genistein; fractions 1, 7 and 21 do not.

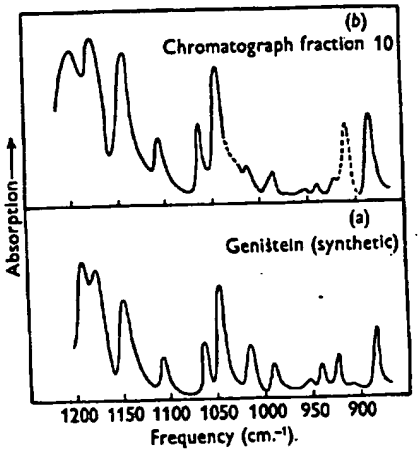

Fig. 3. Infrared absorption curves of (*a*) synthetic genistein and (*b*) chromatograph fraction from subterranean clover. Bands due to impurities in the chromatograph fraction are dotted. (Grubb-Parsons S3A Spectrometer equipped with sodium chloride prism. Solid samples ground with paraffin oil.)

Fractions 9–16 had maxima at 262·5 mμ. and were all similar to the absorption curve of genistein (Fig. 1). On injection in ovariectomized mice, only the fractions 9–16 showed oestrogenic activity, whereas fractions 1–8 and 17–21 were inactive. Recrystallization of fractions 9–16 yielded genistein, identified by its m.p. and u.v. absorption. As a final proof of identity, fraction 10 showed absorption in the infrared region, 860 cm.$^{-1}$ to 1200 cm.$^{-1}$, very similar to that of synthetic genistein, as shown in Fig. 3, with impurities showing absorption at 1030 and 910 cm.$^{-1}$.

In the crude fractions, however, the absorption at the minimum (231 mμ.) was greater than that for pure genistein, whereas the maximum absorption (262·5 mμ.) was low. To compensate for the impurities, an arbitrary line was calculated giving assumed values for the impurities of $E^{1\%}_{1\,cm.}$ (231 mμ.) = 450 and $E^{1\%}_{1\,cm.}$ (262·5 mμ.) = 320. If in Fig. 4 the observed values for the fraction are $e = E^{1\%}_{1\,cm.}$ (231 mμ.) and $f = E^{1\%}_{1\,cm.}$ (262·5 mμ.), then

$$500x + 450n = e \quad \text{and} \quad 1400x + 320n = f,$$

where x is the proportion of genistein present and n the 'proportion' of impurities. From these equations:

$$x = (0{\cdot}96f - 0{\cdot}68e) \times 10^{-3},$$

and

$$n = (2{\cdot}98e - 1{\cdot}06f) \times 10^{-3}.$$

To test the validity of the assumed values for the absorption by impurities, x and n were calculated for a number of fractions and the theoretical curve was drawn for the calculated mixture. There was close agreement between the observed and calculated absorption.

The similarity between the absorption by impurities in the genistein fractions and the absorption by the non-genistein fractions suggested that the same formula may be used to estimate genistein from the curves obtained from the

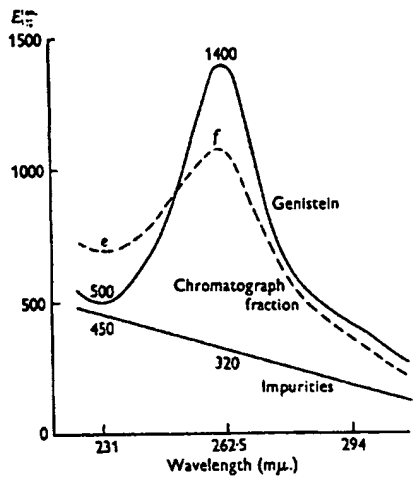

Fig. 4. Arbitrary straight-line absorption assumed due to impurities in chromatograph fractions. From the values shown, the proportion of genistein present in the fraction is calculated as: $x = (0{\cdot}96f - 0{\cdot}68e) \times 10^{-3}$.

whole phenol fraction without chromatography. A typical application of this procedure is demonstrated in Fig. 5, which shows close agreement between the observed and calculated curves.

Thus, by reading the values for $E^{1\%}_{1cm}$ (231 mμ.) and $E^{1\%}_{1cm}$ (262·5 mμ.), the amount of genistein present in the phenols may be calculated. A slide rule was constructed for the purpose of making the calculation. This has been applied to a considerable number of extracts and provides a reasonably accurate method of estimation of genistein in clovers. Similar correction methods have been applied by Morton & Stubbs (1946) in vitamin A determinations and by Sveinsson, Rimington & Barnes (1949) in porphyrin determinations. Genistein added to clover was recovered to the extent of 96–109% in six different estimations.

Micro-method of estimation of genistein in clover

Extraction and purification. Two leaves, of similar age, were taken from each clover plant. Both were weighed, one dried at 100° for the determination of dry matter content, and the other placed in a 3 × ½ in. test tube and refluxed with a cold thimble for 10 min. with 1 ml. ethanol. The ethanol solution was decanted into a second tube graduated at 0·6 and 1·0 ml. The leaf residue was refluxed with a further 0·5 ml. ethanol for 5 min. and the extract decanted into the graduated tube. The combined extracts were evaporated by using a stream of air or nitrogen directed into the tube, keeping the tube warmed to about 70° in a glycerol bath, until the volume was reduced to 0·6 ml. Water was added to bring the volume to 1·0 ml. and 0·5 ml. benzene was added. The tube was rotated at an angle of 45° from vertical in a mechanical rotator at about 140 rev. min.$^{-1}$ for 10 min. This procedure avoided the formation of emulsions and gave an efficient extraction, the benzene layer being dark green in colour whereas the aqueous alcohol layer was almost colourless.

With the use of a pipette inserted to the bottom of the tube, 0·5 ml. of the aqueous phase, free of the benzene phase, was drawn up and a volume equivalent to 2·5 mg. of fresh clover placed in a small tube and evaporated to dryness by the technique described above.

Paper chromatography. Following the method of Bate-Smith & Westall (1950), the extract was dissolved in a minimum of butanol containing 1% HCl (w/v) and transferred with a micro-pipette to a paper strip (Whatman no. 4) 5 mm. wide, care being taken to keep the length of the applied spot within a 5 mm. limit. Control strips were spotted similarly with 2·5 and 5·0 μg. genistein.

The strips were placed in an atmosphere saturated with the vapour of the aqueous phase of a butanol–acetic acid–water mixture (40:10:50, v/v) for 24 hr. The solvent mixture, butanol–acetic acid–water (40:10:20, v/v), was then introduced for ascending chromatography. After the solvent front had moved approx. 30 cm. (18 hr.) the strips were dried in a current of air in the fume hood and sprayed with aqueous 0·1% (w/v) $FeCl_3$ solution, freshly prepared from a 10% stock solution. The pink genistein spot ($R_F = 0·9$) was compared with the control strips.

Throughout a series of several hundred separations it was found impossible to estimate genistein content by measuring spot length. Also, the separation of genistein from the pigments present was not always complete, so that only a visual approximation of genistein content, by comparing the extract chromatograms with the controls, was possible.

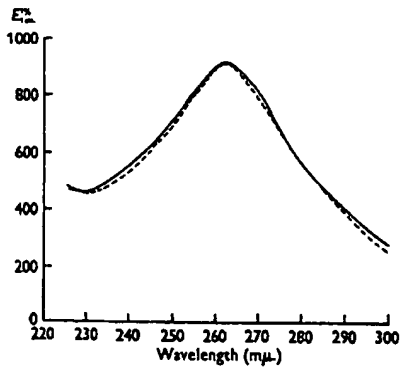

Fig. 5. Observed absorption curve (——) of phenols from subterranean clover (no. P_4) and calculated curve (· · ·). For explanation see text.

RESULTS

The results of a series of determinations on different clovers by using silica gel chromatography and ultraviolet absorption measurements are given in Table 2.

Table 2. *The genistein content of several clovers*

30 g. fresh clover were used and the results are calculated on the basis of 100 g. dry matter.

		Ethanol extract					
			60% ethanol phase				
No.	Description	Benzene phase (g.)	Neutrals (g.)	Acids (g.)	Phenols (g.)	Residue (g.)	Genistein (g.)
1	*T. subterraneum* var. *Dwalganup*	10·3	0·5	0·4	1·6	76·4	0·740
2	*T. subterraneum* var. *Burnerang*	11·3	0·5	0·4	1·4	86·6	0·400
3	*T. subterraneum* var. *Red Leaf*	8·2	0·5	0·2	0·9	76·4	0·550
4	*T. subterraneum* var. *Tallarook*	7·7	0·4	0·5	1·2	85·7	0·565
5	*T. repens*	7·9	0·2	0·1	0·2	75·8	Nil
6	*T. fragiferum*	9·4	0·8	0·5	0·2	73·4	Nil
7	*T. pratense*	15·5	0·5	0·1	0·6	76·6	Nil
8	*Melilotus alba*	9·3	0·4	0·3	0·2	71·2	Nil

The four subterranean clovers contained large amounts of genistein, whereas the others contained no trace of genistein. With the micro-method these studies have been extended to a large number of clovers and the results will be given in a further communication. The genistein content of many samples of the *Dwalganup* strain has now been determined and these results will be available for publication shortly. The figure for *Dwalganup* in Table 2 is a good representative one.

DISCUSSION

The preparation of the 'chloroplast' fraction of clover, although of use in providing a concentrate in readily stored form, is effective in retaining only about 3% of the active compound. Quantitative estimations of the intake of genistein by sheep, based on the amount present in this fraction, have been most misleading.

The isolation of substantial amounts of genistein from fresh subterranean clover, however, makes it likely that this *iso*flavone is responsible for the breeding problem in sheep maintained on pastures dominated by this plant species. A sheep eating 1 kg. of dry matter per day would have an intake of about 7 g. genistein daily. Genistein, on oral administration in mice has a median effective dose (M.E.D.) of 8·2 mg. (Biggers & Curnow, 1954) so the sheep receives about 850 mouse M.E.D.'s of genistein daily. With injected stilboestrol complete infertility is produced in ewes receiving 0·1 mg. or approx. 4000 mouse M.E.D.'s per day for 6 months (Underwood, Shier & Peterson, 1953). It is not unlikely that a degree of infertility equivalent to that often induced by clover grazing (20–60%) would result from the injection of 0·02 mg. (approx. 800 mouse M.E.D.'s) of stilboestrol daily, especially if the treatment were carried on for more than one season, as occurs with clover ingestion. Although it is dangerous to make assumptions regarding the activity of genistein in the sheep from the behaviour in mice, it is reasonable to suppose that a somewhat similar response to that with stilboestrol will be given by the daily 850 mouse M.E.D.'s of genistein. It is possible also that genistein is transformed in the rumen of the sheep to a more active oestrogen. There is evidence (Beck & Curnow, unpublished data) that treatment of clover 'chloroplast' with alkalis yields a small amount of a second oestrogen with at least ten times the activity of genistein.

SUMMARY

1. From the 'chloroplast' fraction of subterranean clover (*Dwalganup* strain) 12 mg. genistein per 100 g. dry matter of clover was isolated.

2. From fresh clover 369 mg. genistein per 100 g. dry matter was isolated.

3. A method of estimation of genistein in plant material and a micro-method of detection of genistein in small amounts of plant material have been developed.

4. Four subterranean clovers studied contain genistein, whereas four other species contained no genistein.

5. The *Dwalganup* strain of subterranean clover contained 740 mg./100 g. dry matter, sufficient to account for the oestrogenic activity of the plant and therefore probably responsible for the infertility in sheep grazing on subterranean clover pastures.

I wish to thank the Western Australian Investigational Committee, Professor E. C. Dodds, Dr C. P. Rhoads and the late Dr K. Dobriner for generous assistance in many ways, also Dr A. R. H. Cole for the infrared spectrometry, Dr Lawrence for the use of the ultraviolet spectrophotometer and Mr K. Gillieatt for able technical assistance.

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Use of a Mammalian Cell Culture Benzo(*a*)pyrene Metabolism Assay for the Detection of Potential Anticarcinogens from Natural Products: Inhibition of Metabolism by Biochanin A, an Isoflavone from *Trifolium pratense* L[1]

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ABSTRACT

Based on the epidemiological evidence for a relationship between consumption of certain foods and decreased cancer incidence in humans, an assay was developed to screen and fractionate plant extracts for chemopreventive potential. This assay measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene [B(a)P] in hamster embryo cell cultures. Screening of several plant extracts has generated a number of activity leads. The 95% ethyl alcohol extract of one of these actives, *Trifolium pratense* L. Leguminosae, red clover, significantly inhibited the metabolism of B(a)P and decreased the level of binding of B(a)P to DNA by 30 to 40%. Using activity-directed fractionation by solvent partitioning and then silica gel chromatography, a major active compound was isolated and identified as the isoflavone, biochanin A. The pure compound decreased the metabolism of B(a)P by 54% in comparison to control cultures and decreased B(a)P-DNA binding by 37 to 50% at a dose of 25 μg/ml. These studies demonstrate that the hydrocarbon metabolism assay can detect and guide the fractionation of potential anticarcinogens from plants. The ability of the isoflavone biochanin A to inhibit carcinogen activation in cells in culture suggests that *in vivo* studies of this compound as a potential chemopreventive agent are warranted.

INTRODUCTION

Humans are exposed to numerous carcinogens and mutagens daily, some avoidable (such as cigarette smoking) and some virtually unavoidable (diet, environmental pollution, oxygen radicals). The diet has been shown to have a profound effect on the incidence and location of various human cancers worldwide (1, 2), and epidemiological studies suggest that certain dietary components may help to prevent cancer induction. This prophylaxis has been termed cancer chemoprevention. Wattenberg (3) has demonstrated that such agents may inhibit cancer induction by a number of mechanisms. One of the more common mechanisms is through inducing alterations in the enzymatic activation or detoxification of carcinogens.

Although many biological assays have been used to examine the chemopreventive potential of various chemicals, there have been relatively few studies using activity-directed fractionation to isolate active compounds from plants. In addition, it is impractical to use *in vivo* models to guide these procedures. Loub *et al.* (4) used an activity-directed fractionation procedure based upon induction of aryl hydrocarbon hydroxylase activity in the liver and intestinal mucosa of Sprague-Dawley rats to isolate and identify several indoles from cruciferous vegetables. Kaweol and caffestol palmitates were isolated from green coffee beans (5) based upon an assay that measured the increase in glutathione S-transferase activity in liver and intestinal mucosa of mice. Practical assays for activity-directed fractionation of active plants must be rapid, sensitive, convenient, and capable of detecting alterations in carcinogen metabolism. In this paper, we describe the development and application of an assay that measures effects on the metabolism of [^{3}H]benzo(*a*)pyrene, a widespread environmental carcinogen, in early passage cultures of Syrian hamster embryo cells (6). The chemical and analytical procedures developed for activity-directed fractionation of antineoplastic compounds from plants (7, 8) were adapted to the isolation and identification of potential anticarcinogens from food and food plants, such as red clover extracts, which significantly inhibited the metabolism of benzo(*a*)pyrene and binding of B(a)P[3] metabolites to DNA.

MATERIALS AND METHODS

Spectroscopy and Chromatography. ^{1}H NMR in deuteriochloroform was performed using a Varian XL-200, and ^{13}C NMR in deuteriochloroform was measured on a Chemagnetics A-200 spectrometer. EI and CI mass spectra were obtained on a Finnigan 4023 quadrupole mass spectrometer. High-resolution mass spectra were recorded on a Kratos MS 50. The IR spectrum was performed on a Beckman IR-33 using a KBr pellet. UV spectra were measured on a Beckman DU-7 in methyl alcohol using sodium methoxide, $AlCl_3$, HCl, and sodium acetate as UV shift reagents.

For flash column chromatography EM 9385 Silica Gel 60 was used for the adsorbent. Radial chromatography was performed on a Chromatotron Model 7924 using a 1-, 2-, or 4-mm rotor with EM 7749 Silica Gel 60 PF 254 as adsorbent. TLC plates were Merck 5714 Silica Gel 60 F_{254}.

Cell Culture Toxicity Assay. Hamster embryo cell cultures were prepared and grown as described previously (6). Tertiary cultures were plated in 60-mm plastic dishes (Falcon) (5×10^5 cells), and 24 h later the test compound was added at 10-fold dilutions from 500 μg/ml of medium to 0.05 μg/ml for 24 h. At that time the cultures which were approximately 70% confluent were examined microscopically and subjectively evaluated for the percentage of the cells dividing and the cell density. The highest noninhibitory dose was selected for metabolism studies.

B(a)P Metabolism Assay. Tertiary hamster embryo cell cultures (10^5 cells per 25-cm^2 flask, 3 flasks per group) were plated in 8 ml of medium containing 10% calf serum and refed with 8 ml of fresh medium after 48 h. Seventy-two h after plating, the cultures were treated with the test compound in DMSO or DMSO as a control, and 30 min later [^{3}H]-B(a)P (1 μg/ml; specific radioactivity, 0.25 Ci/mmol) was added. Twenty-four h later medium was removed and stored at −20°C. Aliquots (0.2 ml) were extracted by a two-stage chloroform:methanol:water procedure (6, 9). The assay uses initial mixing with a vortex mixer in a single-phase system of chloroform:methanol:water (including the medium) (1:2:0.8) to ensure complete extraction of the lipophilic hydrocarbon and its metabolites followed by addition of 1 ml of chloroform

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[3] The abbreviations used are: B(a)P, benzo(*a*)pyrene; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; MS, mass spectrum; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; B(a)PDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; dGuo, deoxyguanosine.

and 1 ml of water and mixing with a vortex mixer. After centrifugation for 10 min, the aqueous phase was removed and extracted with 2.0 ml of chloroform to ensure complete extraction. The chloroform extracts were then pooled, and the radioactivity in the organic and aqueous-methanol phases was measured by liquid scintillation counting of 0.1-ml aliquots. This extraction procedure results in recovery of unmetabolized B(a)P and Phase I metabolites (dihydrodiols, quinones, and phenols) in the chloroform phase. The water-soluble metabolites including glucuronides and glutathione conjugates and multiple oxidation products are retained in the aqueous-methanol phase. Since the large majority of the metabolites formed from B(a)P in hamster embryo cells are water soluble (usually greater than 80%) (6), this assay provides a rapid measure of B(a)P metabolism.

BHA, a known inhibitor of carcinogenesis and B(a)P metabolism (10), was used to treat a positive control group in all assays at a concentration of 50 μg/ml of medium. The highest nontoxic dose of BHA was selected from multiple experiments using different hamster embryo cell preparations. Doses of 75, 65, 50, and 5 μg/ml of medium were tested, and the results show that 75 μg/ml were toxic and 65 μg/ml exhibited borderline toxicity, while 50 μg/ml showed a significant inhibition of B(a)P metabolism with no cell toxicity. The lowest dose, 5 μg/ml, produced no significant inhibition of B(a)P metabolism. Using BHA as a positive control gave us an indication of the health and viability of the cells in the culture assay for that particular experiment and helped eliminate false negatives.

Analysis of B(a)P Metabolites. The B(a)P metabolites in the organic phase were analyzed by HPLC on an Ultrasphere C_{18} column (25 cm × 4.6 mm) eluted with a methanol:water gradient as described previously (6). UV absorbing standards of authentic B(a)P metabolites (Chemical Repository, Division of Cancer Etiology, National Cancer Institute) were included in each HPLC analysis. The radioactivity was monitored with a Flo-one β flow monitor set to update every 30 s.

Binding of B(a)P to DNA. Tertiary hamster embryo cell cultures (5×10^7 cells) were plated in 175-cm^2 flasks containing 50 ml of minimal essential medium with 10% fetal bovine serum. After 2 days the cultures were refed with fresh medium and 24 h later with the test compound, or extract in DMSO was added. Five to 10 min later the cultures were treated with [^{3}H]B(a)P (1 μg/ml of medium, 0.5 mCi/flask). After 24 h of incubation at 37°C the cells were harvested, and DNA was isolated as described previously (11). The radioactivity in an aliquot was measured by liquid scintillation counting, the amount of DNA was determined by A_{260}, and these values were used to calculate the level of B(a)P metabolites bound to DNA.

After enzymatic degradation of the DNA to deoxyribonucleosides, the B(a)P:deoxyribonucleoside adducts were isolated by chromatography on Sep-Pak C_{18} cartridges and analyzed by HPLC on a 25-cm × 4.6-mm Ultrasphere C_8 reversed-phase column (11). The column was eluted at a flow rate of 1.0 ml/min with methanol:water (46:54) for 34 min, a linear gradient for 10 min (46:54 to 55:45) and at 55:45 for 24 min. Fifteen 1.0-ml fractions followed by 165 fractions (0.3 ml) were analyzed by scintillation counting.

Plant Extraction. Leaves, stems, and flowers of *Trifolium pratense* L (red clover) were collected. A voucher specimen is on deposit in the biology herbarium of the Department of Biology, Purdue University. The fresh plant (918 g) was ground with 2 liters of 95% ethyl alcohol in a commercial size Waring blender for 5 min. The blended material was then allowed to stand for 30 min to complete the extraction. The material was then filtered through a Büchner funnel, and the filtrate was concentrated *in vacuo* to give 46.5 g of the 95% ethyl alcohol extract. An aliquot was dissolved in DMSO and submitted for testing. The 95% ethyl alcohol extract was found to be active and therefore was then further partitioned according to the scheme shown in Fig. 1. The testing data are shown in Table 1. All fractions were tested at the dose-response dose which was defined as the percentage of the 95% ethanol-extractable material that the fraction represented times the dose of 95% ethanol fraction used in the metabolism assay (in this case, 750 μg/ml of medium).

Isolation and Identification of Active Components. Aliquots from the solvent partition were submitted for testing. The active $CHCl_3$ fraction was subjected to silica gel flash column chromatography with hexane, $CHCl_3$, ethyl acetate, acetone, acetone:methyl alcohol (1:1), and finally methyl alcohol. Nine fractions were collected, and aliquots were taken and submitted for testing. The column fraction which was active at the dose-response dose (Fraction 1D) (see Fig. 1) was further chromatographed by centrifugal silica gel TLC (Chromatotron) using a $CHCl_3$/methyl alcohol solvent gradient starting with 2% methyl alcohol in $CHCl_3$. The fractions which were collected were combined according to the presence of similar spots when analyzed by silica gel TLC developed in 2% methyl alcohol in $CHCl_3$. Based upon this, the samples were combined into seven fractions which were tested for their effects on B(a)P metabolism. The most active fraction (2D) was further separated on another silica gel Chromatotron plate developed in a $CHCl_3$/methyl alcohol gradient. Based upon TLC profiles eluants were combined into three fractions. The most active fraction (3B) contained a major component. Recrystallization of this fraction from aqueous methyl alcohol gave a crystalline material, m.p. 217–218°C. A sample of authentic biochanin A was purchased from Aldrich Chemical Co., m.p. 218–219°C. A mixed m.p. showed no depression. The UV and ^{1}H NMR data were identical to literature values (12), and the MS and ^{13}C NMR data were consistent with the published structure.

Examination of the interface fraction, which was active at 2× the dose-response dose, led to the isolation of additional biochanin A, along with an analogue, formononetin (see Fig. 3). Formononetin was inactive in the metabolism bioassay. Biochanin A represented about 30% of the interface fraction.

RESULTS

The results of bioasssay-directed fractionation of the active ethyl alcohol extract of red clover are presented in Fig. 1 and Table 1. The ethyl alcohol extract was active at doses from 500 μg/ml to 1000 μg/ml; however, toxicity was detected at the highest dose (see Table 1). Further partitioning of the active ethyl alcohol extract was dose responded from 750 μg/ml. After partitioning between chloroform and water, the activity appeared in the chloroform extract. Examination of the interface which was active at twice the dose-response dose confirmed the presence of biochanin A. Chromatography of the chloroform fraction gave active column Fraction 1D. This fraction was carried through two separations on the Chromatotron to give in turn active Fractions 2D an 3B. Crystallization of Fraction 3B gave 30 mg of the active constituent, biochanin A. Fractions 1A, 1B, 2B, and 2F show activity at twice the dose-response dose and are under further investigation. The B(a)P metabolites present in the organic phase of the sample treated with red clover extract at 500 μg/ml were analyzed by HPLC, and the amount of the major primary B(a)P metabolites was determined (Fig. 2). The two major changes were a slight increase in the amount of 9-hydroxy-B(a)P and a major decrease in the amount of water-soluble metabolites in the extract-treated group. After β-glucuronidase treatment of the aqueous phase, the amount of 9-hydroxy- and 3-hydroxy-B(a)P in the red clover extract-treated group was reduced by 30% and 22%, respectively, when compared to DMSO controls. The water-soluble metabolites were also decreased by 18% in the red clover extract-treated cells. Thus the major effect of red clover extract was to inhibit the formation of B(a)P-phenol glucuronides.

The effect of the crude 95% ethyl alcohol extract on the binding of B(a)P to DNA was also examined (Table 2). At a dose of 250 μg/ml the extract inhibited B(a)P-DNA binding by 30% to 41% comparred to controls in three separate experiments. Analysis of the B(a)P-DNA adducts present in enzyme-digested DNA samples by HPLC demonstrated that the extract inhibited the formation of both the *syn*- and *anti*-isomers of B(a)PDE. The *syn*-B(a)PDE-dGuo adducts decreased from 37% to 64% compared to controls, and the (+)-*anti*-B(a)PDE-dGuo adduct decreased from 48% to 75%.

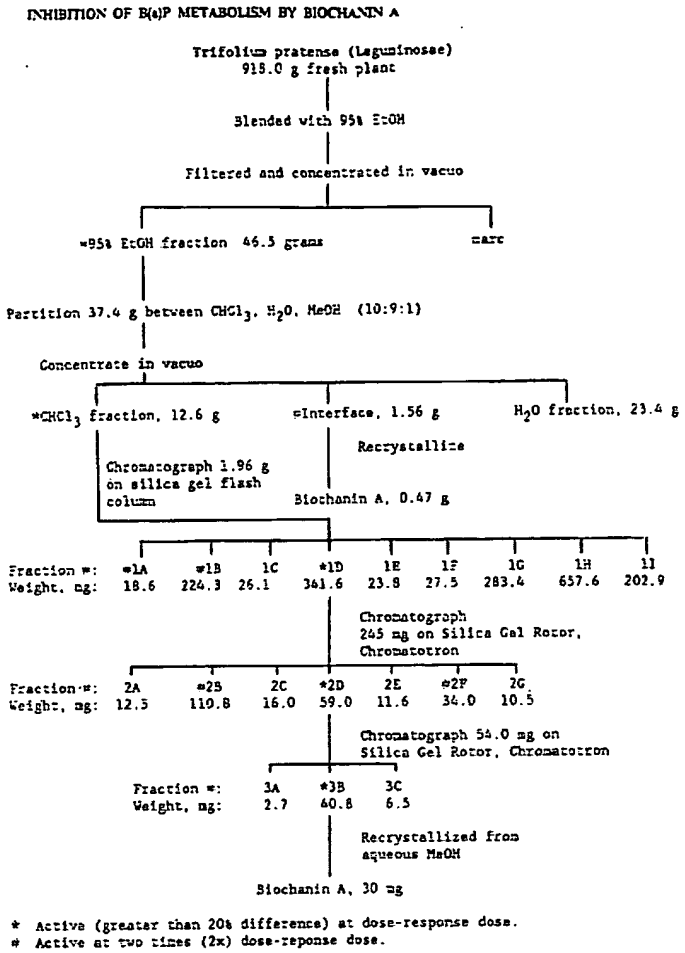

Fig. 1. Fractionation scheme for the 95% ethyl alcohol extract of fresh red clover leaves, flowers, and stems. *EtOH*, ethyl alcohol; *MeOH*, methyl alcohol.

The effect of biochanin A on the binding of B(a)P to DNA in hamster embryo cell cultures was also examined. Biochanin A caused a 54% decrease in B(a)P metabolism at 25 μg/ml. After exposure of cultures to 25 μg of biochanin A and 1 μg of [^{3}H]B(a)P per ml of medium for 24 h, biochanin A treatment reduced the amount of B(a)P bound per mg of DNA from 74.3 pmol in the control group to 35.1 pmol in the biochanin A group in one experiment and from 72.2 to 45.4 in a second experiment. Thus, biochanin A inhibited the binding of B(a)P to DNA to an extent similar to that obtained in the crude extract (Table 2).

DISCUSSION AND CONCLUSIONS

There are several bioassays which are under investigation for the detection of compounds suspected of having potential cancer chemopreventive activity. Antimutagenic activity in the form of an anti-Ames assay has been commonly used in the United States and Japan (13, 14). Mitscher *et al.* (15) used this bioassay to isolate and identify glabrene, a known isoflavene exhibiting antimutagenic activity. Nishino *et al.* (16) used the antitumor-promoting activity of glycyrrhetic acid against 7,12-dimethylbenz(a)anthracene and teleocidin as a model of cancer prevention. The decrease in formation of carcinogenic *N*-nitroso compounds produced by α-tocopherol and ascorbic acid was used as a criterion for chemoprevention by Narkus *et al.* (17) and Mervish (18). Sakiyama *et al.* (19) used the inhibition of transformation of the mouse 10T½ cell line induced by X-ray or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a model to show the anticarcinogenic effects of lipopolysaccharides and indomethacin. The induction of aryl hydrocarbon hydroxylase activity in liver and intestinal mucosa of Sprague-Dawley rats was used by Wattenberg *et al.* (3) to isolate and identify a group of indoles from cruciferous vegetables (20, 21). Another screen by Wattenberg *et al.* (5) used the induction of glutathione *S*-transferase activity, a major detoxification enzyme system, for a number of electrophiles, including many carcinogens, in mouse liver and intestinal mucosa to isolate a group of known diterpenes from green coffee beans.

The screening procedure described in this paper measures effects on the ability of hamster embryo cell cultures to metabolize the carcinogen B(a)P. Induction of inhibition of B(a)P metabolism of treated cultures by >20% as compared with control cultures was considered to be an active test. The altered pattern of metabolism was determined by HPLC analysis of the B(a)P metabolites formed, and the effects on binding of B(a)P to DNA are determined. Confirmed active extracts are then fractionated using the bioassay as a guide. Advantages of our method are that activity data can be generated within a few days after the extract or compound is tested, and a large number

Table 1 *Activity of fractions in B(a)P metabolism assay*

The procedure used for analysis of B(a)P metabolism to water-soluble metabolites is described in "Materials and Methods."

Fraction	Dose-response dose (μg/ml medium)	% of change from control	2× dose-response dose	% of change from control
95% Ethyl alcohol	500	−39.9 ± 6.2[a, b]		
	750	−40.4 ± 6.9[b, c]		
$CHCl_3$	203	−35.6 ± 5.7[b, c]		
Interface	25.2	−17.7 ± 7.3[c]	406	−68 ± 4.4[c]
H_2O	541	−15.4 ± 1.4[c]	50.2	−33.8[c, e]
1A	1.9	17.2 ± 5.4[a]		
1B	23.3	−5.9 ± 15.2[a]	3.9	28.8[c, e]
1C	3.7	8.7 ± 5.6[a]	46.6	−34.6 ± 4.6[c, e]
1D	35.5	23.5 ± 4.0[b, c]	7.4	−8.0[d]
1E	2.5	3.25 ± 6.8[c]	71.0	−50.2[c, e]
1F	2.8	8.5 ± 0.8[c]	5.0	−3.5[d]
1G	29.4	12.1 ± 7.3[c]	5.6	13.6[d]
1H	68.1	5.0 ± 9.2[c]	58.8	17.8[d]
1I	21.0	−5.9 ± 11.4[c]	136.2	−5.5[d]
2A	1.8	−14.1[d]	42.0	−11.7[d]
2B	16.1	−12.9[d]	3.6	4.8[d]
2C	2.3	−13.8[d]	32.2	−32.7[c, e]
2D	8.6	−25.3[b, d]	4.6	−12.9[d]
2E	1.7	−13.0[d]	17.2	−19.9[d]
2F	4.9	−14.6[d]	2.4	3.0[d]
2G	1.5	−1.8[d]	9.8	−23.0[c, e]
3A	0.4	−6.8[d]	3.0	
3B	6.4	−23.7[b, d]	0.8	
3C	1.0	5.93 ± 18.6[e]	12.8	−23.0 ± 22.9[c, f]
Biochanin A	4.7	−12.2[d]	2.0	8.8[d]
	9.5	−32.1[d]		
	19.0	−47.4[d]		
	23.6	−48.8[d]		

[a] Mean ± SD of 3 experiments.
[b] Active (greater than 20% difference) at dose-response dose.
[c] Average ± range of 2 experiments.
[d] One experiment.
[e] Active at 2× dose-response dose.
[f] Mean ± SD of 4 experiments.

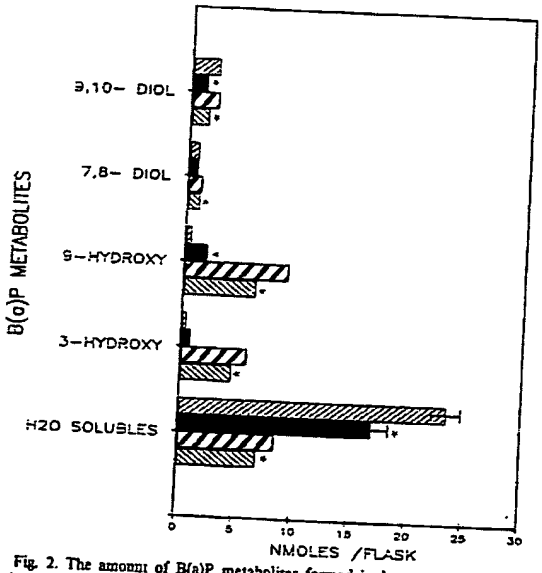

Fig. 2. The amount of B(a)P metabolites formed in hamster embryo cell cultures in the presence or absence of 500 μg/ml of red clover crude extract. The cultures were treated, and the medium samples used analyzed as described in "Materials and Methods." Medium samples were treated with β-glucuronidase prior to extraction to determine glucuronide conjugates. *Columns*, mean for 3 flasks per group; *bars*, SD. *, red clover extract-treated samples that differed significantly from the corresponding control (based upon Student's *t* test; $P < 0.01$). ▨, control; ■, *T. pratense*; ▨, control (β-Glucuronidase); ■, *T. pratense* (β-Glucuronidase).

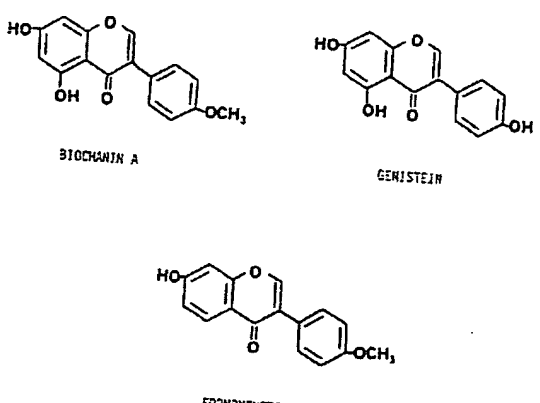

Fig. 3. Isoflavones isolated from red clover leaves and flowers (25, 26).

of different samples can be screened simultaneously. After pure active compounds are isolated and their effect on the metabolic activation of B(a)P is established, they will then be further tested using *in vivo* bioassays to determine their effect on tumor induction by various classes of carcinogens. These *in vivo* bioassays are essential for determining whether a compound acts as an anticarcinogen and against which classes of carcinogens it was active.

Thus far we have screened over 70 species and varieties of plants and vegetables comprising 27 families. One of the first plant extracts demonstrated to produce reproducible inhibition of B(a)P metabolism in the hamster cell culture assay was that prepared from red clover. Based upon inhibition of B(a)P metabolism the crude red clover extract was fractionated, and

Table 2 *B(a)P-DNA binding in hamster embryo cells treated with extracts of red clover*

Hamster embryo cell cultures were exposed to the 95% ethyl alcohol extract of red clover at a dose of 250 μg/ml of medium, and 10 min later 1.0 μg of [^{3}H] B(a)P per ml of medium was added. After 24 h, the medium was removed, and a sample was analyzed by chloroform:methanol extraction as described in "Materials and Methods." The percentage of radioactivity in the water phase is reported as the percentage of water-soluble B(a)P metabolites. The DNA was isolated from the cells, and the level of binding of B(a)P was measured. The DNA was digested to deoxyribonucleosides, and the amount of the major B(a)P-DNA adducts was determined by HPLC.

	Experiment 1		Experiment 2		Experiment 3	
	Control	Test extract	Control	Test extract	Control	Test extract
% of water-soluble B(a)P metabolites	48.4	40.5	30.8	15.4	35.6	27.5
Binding of B(a)P to DNA; total level of binding (pmol/mg DNA)	67.0	42.7	51.2	30.0	23.0	15.8
(+)-*anti*-B(a)PDE-dGuo adduct (pmol/mg)	12.7	4.9	11.1	2.7	5.5	2.9
syn-B(a)PDE-dGuo adduct (pmol/mg)	8.4	3.0	8.1	3.7	5.0	3.3

a pure active compound, biochanin A, was isolated which produced an inhibition of B(a)P metabolism of 30 to 50% at 9.5 to 23.6 μg/ml compared to DMSO controls. Exposure of hamster embryo cell cultures to biochanin A at a dose of 25 μg/ml of medium resulted in a 37 to 50% inhibition in the binding of B(a)P to DNA. This compound appears to be one of the major components responsible for the inhibition of B(a)P-DNA interactions by the red clover extract. The strong correlation between the binding of aromatic hydrocarbons to DNA and their carcinogenic activity suggests that biochanin A is a good candidate for further testing to measure inhibition of tumor induction by hydrocarbons in animals.

Several flavonoids have been shown to possess anticarcinogenic activity (3). 7,8-Benzoflavone, a synthetic flavonoid, is an inhibitor of microsomal mixed-function oxidases and inhibits the metabolism, binding to DNA, and tumorigenesis of 7,12-dimethylbenz(*a*)anthracene in mouse skin (22). This same flavonoid also inhibits the metabolism of B(a)P in rat hepatic microsomes that have been induced with 3-methylcholanthrene (23). Huang *et al.* (24) examined 28 flavonoids for their effect on mutagenicity of anti-B(a)PDE in *Salmonella* and found that 8 had significant antimutagenic activity. Interestingly one of the flavonoids found to be inactive (50% inhibitory dose > 100) was genistein, an isoflavone related to biochanin A (see Fig. 3) and a minor constituent of red clover (25, 26). Since the compound tested [B(a)PDE] was an ultimate mutagenic metabolites of B(a)P, that assay would not be expected to detect compounds that alter metabolic activation of B(a)P. Thus, various types of short-term assays may be anticipated to detect anticarcinogens that work by different mechanisms. In view of the requirement of the majority of classes of chemical carcinogens for metabolic activation and the ability of the metabolism assay to measure changes in enzymes both involved in activation as well as detoxification, the hamster cell assay should be capable of detecting modifiers of carcinogen metabolism that act by a number of mechanisms. The results demonstrate that the effects of test compounds on B(a)P metabolism and DNA binding in hamster embryo cell cultures can be used to screen and isolate pure compounds with potential anticarcinogenic activity from plants and other natural products.

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Naturally occurring oestrogens in foods—A review

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This review is concerned with the presence of naturally occurring oestrogens in food plants and processed foods. Particular emphasis is placed on isoflavones and coumestans, both of which are true plant oestrogens, and the resorcylic acid lactones, more correctly classified as fungal oestrogens. The metabolism and mode of action of these compounds is discussed and their biological potencies, determined in both *in vivo* and *in vitro* studies, described. Current methods of analysis are indicated and the levels of these oestrogens in food plants, processed foods and feedingstuffs are presented. Botanical, environmental or technological factors affecting the possible intake of plant and fungal oestrogens are mentioned and the hazard associated with such intake is compared with that originating from other dietary or medicinal hormonally active substances. Indications are given of the wide range of common food plants which have been reported to possess oestrogenic (uterotropic) activity, although it is emphasized that in general further work is necessary to substantiate these claims and to confirm the identities of the biologically active principles which have in some cases been proposed. In the concluding section suggestions are made for additional research considered important or necessary in this interesting area.

Introduction

The presence in plants of oestrogens, compounds which induce oestrus in immature animals or interfere with normal reproductive processes, has been known for over half a century. However, the use of plants and plant extracts to control fertility in animals and humans has been recognized since earliest times. In the Orient, for example, the pomegranate has traditional associations with fertility which stretch back over 2000 years. Although many of the plant oestrogens have now been separated, purified and characterized, only occasionally have they been found to be identical with those of animal origin, oestrone (I) and 17β-oestradiol (II) (Hewitt *et al.* 1980) (see figure 1).

In 1954, Bradbury and White listed 53 plants which possessed the capacity to initiate oestrus in animals, but progress in this area was such that only two decades later Farnsworth *et al.* (1975) were able to describe over 300 such plants. In many cases the exact nature of the active principles has not been established but, of the identified compounds, isoflavones and coumestans are the most common. In all, these authors listed 29 plant oestrogens, many of which possessed structural similarity to synthetic diethylstilboestrol (III) (figure 1). Less than half the compounds listed have been reported in plants which are regularly consumed by animals or man. Such plants are listed in table 1 and it may be noted that certain of these, for example legumes and fodder crops, may be consumed in relatively large amounts. Indeed, problems of infertility in livestock (especially sheep) resulting from the grazing of oestrogen-rich pasture or fodders are a serious economic problem in many parts of the world (Hanson *et al.* 1965, Bickoff 1968, Shutt 1976) and have provided the stimulus for much of the

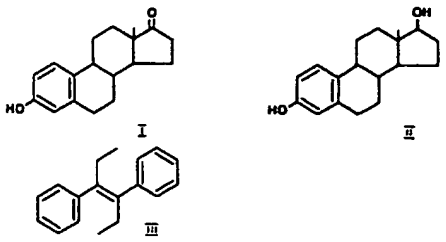


Figure 1. Structures of animal and synthetic oestrogens.

Table 1. Oestrogenic principles of edible plants.

Plant	Common name	Part	Active principle
Avena sativum	oats	seed, meal, sprouts	zearalenone[a] zearalenol[a]
Cicer arietinum	chick pea	seed, seedling	isoflavones
	Bengal gram		isoflavones
Daucus carota var. *sativa*	carrot		3-methyl-6-methoxy-8 -hydroxy,3,4-dihydro isocoumarin[b]
Foeniculum vulgare	fennel	oil	anethole[b]
Glycyrrhiza glabrata	liquorice	root	oestriol, β-sitosterol[b]
Hordeum vulgare	barley	embryo	zearalenone[a]
Humulus lupulus	hops		colupulon[b]- lupulon[b] adlupulon[b]
Malus sylvestris	apple	fruit	oestrone
Medicago hispida	toothed medic		isoflavones
Medicago litteralis	barrel medic		coumestrol
Medicago sativa	alfalfa		4-methoxycoumestrol
Oryza sativa	rice	seed, embryo	zearalenone[a] oestrone, oestradiol
Phaseolus vulgaris	French bean	seedling	oestradiol
Phoenix dactylifera	date palm	seed	oestrone
Pimpinella anisum	anise	oil	anethole[b]
Poa pratensis	bluegrass		isoflavones
Prunus avium	cherry	fruit	prunetin
Punica granatum	pomegranate	seed	oestrone
Secale cereale	rye		zearalenone[a]
Sesamum indicum	sesame	meal	zearalenone[a]
Soja max	soya	seed	isoflavones
		sprouts	coumestrol
Sorghum vulgare	sorghum		zearalenone[a]
Triticum vulgare	wheat	flour, seed, germ oil	zearalenone[a]
Trifolium spp.	clovers	leaves	coumestrol
		stems	isoflavones
Vigna sinensis	cowpea		coumestrol
Zea mays	corn		zearalenone[c] zearalenol[a]
	hay		zearalenone[a]

[a] It should be emphasized that zearalenone and zearalenol are not produced by the plant *per se* but may occur on the plant as a result of synthesis by *Fusaria*.
[b] Tentative.

work which has been conducted on plant oestrogens. However, with the exception of those topics of relevance to the presence and effects of plant oestrogens in the human diet, e.g. studies on the analysis and metabolism of oestrogens and of their possible carry-over into the human body via the ingestion of animal products, the role of such compounds in fodders and other animal feedingstuffs will not be considered here.

Additional interest in naturally occurring oestrogens has resulted from the disquiet of scientists, consumers and legislators over the presence in meat and meat products of compounds, such as diethylstilboestrol, designed to improve animal growth and performance (Umberger 1975, McMartin *et al.* 1978). Although the biological activities of such compounds, expressed on a unit weight basis, are very much greater than those of plant oestrogens (see below), under normally regulated conditions their intake into the human body will be very much less. Since any health risk due to dietary factors is a consequence of both biological potency and exposure, there has in recent years been considerable study of plant oestrogens, their metabolism, modes of action and potencies. Such studies have revealed the considerable extent to which genetic, botanical and environmental factors determine the contents of these compounds and also how the processing of the raw plant prior to its consumption can exert similar effects. These studies have, in no small part, benefitted from the development of improved methods of chemical analysis, possessing greatly improved sensitivity and specificity. This paper reviews the more recent advances in these areas and identifies others awaiting additional investigation.

The major plant oestrogens

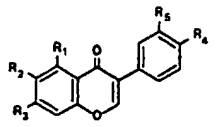
In this review, for convenience, the term 'plant oestrogen' will be used to describe all of the compounds considered in this section, although the resorcylic acid lactones have been referred to elsewhere as *fungal* oestrogens.

The biological effects of plant oestrogens (in the form of the purified compounds or as fresh plants, extracts or processed material) are generally demonstrated by measuring the uterine enlargement of immature female mice or the degree of cornification of the vaginal epithelium. Whereas the former is the more sensitive it lacks the specificity of the latter (Stob 1983); both assays are, however, subject to criticism and misleading results are possible (Emmens 1969).

Examination of table 1 reveals that compounds responsible for the oestrogenic activity mainly fall into three groups, according to their chemical structure. These are (*a*) isoflavones, which in many cases are present in the bound, glycosidic form; (*b*) coumestans; and (*c*) resorcylic acid lactones. A distinction can readily be made between the first two groups and the latter; isoflavones and coumestans are intrinsic plant components, although their levels are dependent upon many factors, including those associated with growth and genetic background. In addition, their levels may also be increased as a direct response to microbial or insect damage. In contrast, the resorcylic acid lactones are products not of the plant *per se*, but of *Fusarium* moulds which are common in the field and flourish in the warm, moist conditions of badly stored grains and other produce. Although other individual compounds possessing oestrogenic activity do occur in food plants, and are considered in the penultimate section of this paper, the major part is concerned with the above groups which are considered separately below.

Isoflavones and isoflavone glucosides

The naturally occurring isoflavones which have been shown to possess oestrogenic activity are (figure 2): daidzein (IV) and genistein (V), their glucosides, daidzin (VI) and



R_1	R_2	R_3	R_4	R_5	
H	H	OH	OH	H	IV
OH	H	OH	OH	H	V
H	H	O-glu	OH	H	VI
OH	H	O-glu	OH	H	VII
H	H	OH	OCH_3	H	VIII
OH	H	OH	OCH_3	H	IX
OH	H	OH	OCH_3	OH	X
OH	H	OCH_3	OH	H	XI
H	H	O-6′acetylglu	OH	H	XII
OH	H	O-6′acetylglu	OH	H	XIII
H	OCH_3	OH	OH	H	XIV
H	OCH_3	O-glu	OH	H	XV

Figure 2. Structures of naturally occurring isoflavones.

genistin (VII), and their 4′-methyl ethers, formononetin (VIII) and biochanin A (IX), respectively; two other active isoflavones, pratensein (X) and prunetin (XI) are of rather limited occurrence. It is possible that other derivatives may also possess uterotropic activity; for example, Japanese workers have isolated the 6′-O-acetyl derivatives of both daidzin and genistin (XII and XIII, respectively) from soyabeans, but they do not appear to have been assayed for their oestrogenic effects (Ohta *et al.* 1979, 1980). It is, however, likely that they are metabolized *in vivo* by ruminants and other animals to daidzin and genistin or their aglycones. Most of the above isoflavones occur in the intact plant in the bound form, as glucosides, but are readily degraded to the aglycone chemically or enzymically during processing, isolation and analysis. Bound isoflavones in clover and related pastures are readily hydrolysed by endogenous glycosidases when the intact plant is crushed (Beck 1964) and such hydrolysis can also occur in animals, and presumably man, in the absence of the plant enzyme. A large number of isoflavones have been isolated from plant species, but only a small number have been shown to possess oestrogenic activity. Moreover, not all isoflavones isolated from plants known to affect oestrus are active; for example, whilst soyabeans possess daidzein, genistein and their glycosides, they may also contain the uterotropically inactive glycitein (XIV) and glycitein-7β-glucoside (XV) (figure 2) (Naim *et al.* 1973).

The biological potencies of the individual isoflavones vary, but all are much less active than animal or synthetic oestrogens. Thus, although genistein is the most potent isoflavone in terms of its effect on mouse uterus (figure 3), it exhibits only 10^{-5} of the activity of diethylstilboestrol. The relative activities of the individual isoflavones vary with both the species and strain of animal used and with the route of administration. In sheep, biochanin A and genistein were about 20 times less active when introduced

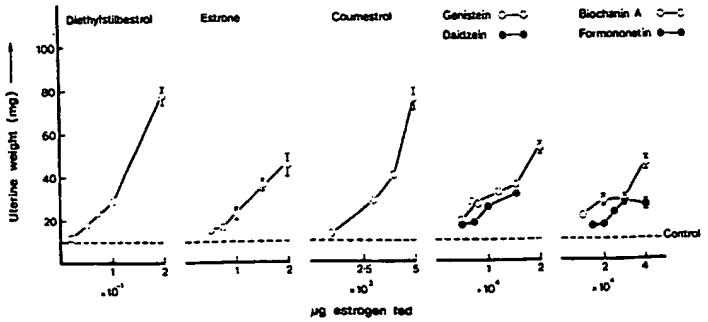


Figure 3. Relative uterotropic potency of diethylstilboestrol, oestrone, coumestrol and isoflavone oestrogens (after Stob 1983).

intraruminally as compared to intramuscular injection, whereas the latter route showed formononetin to be inactive (Braden *et al.* 1976). Genistein was found to be the most active of the isoflavone aglucones tested by oral administration in the mouse (Bickoff *et al.* 1962) and together with its glucoside was equally active when administered subcutaneously (Cheng *et al.* 1955). Differences in responses to other oestrogens between strains of mouse have, however, been reported (Fredericks *et al.* 1981) and recently Farmakalidis and Murphy (1984b) have shown the CD-1 mouse strain to be relatively insensitive to daidzin, genistin and genistein. Comparisons between data arrived at using different strains of mouse are, as the authors point out, thus to be treated with caution. Moreover, there would seem an obvious need to specify, and indeed standardize, the strain of mouse used in the uterotropic assay. Bickoff *et al.* (1962) have demonstrated that dietary isoflavones (daidzein, genistein) possessing a free 4′-hydroxyl group were more uterotropically active in the mouse than their 4′-methyl ethers (formononetin and biochanin A, respectively). The greater potency of genistein compared with daidzein has been attributed to interaction between the OH group and the adjacent carbonyl group of the latter (Bradbury and White 1954). The effect of pratensein is not included in figure 3, but it has been considered to be lower even than formononetin (Wong 1963).

Isoflavones, like the other main groups of plant oestrogens, exhibit an affinity for oestrogen receptor sites (Shutt and Cox 1972, Shutt 1976) and may therefore be considered to function as anti-oestrogens (Martin *et al.* 1978, Verdeal *et al.* 1980). (Anti-oestrogens are thought to exert their effect by decreasing the concentration of cytoplasmic oestrogen receptor and by complexing with the receptor, thus preventing biosynthetic processes associated with tissue development.) The affinities for the binding of genistein to rat, rabbit and sheep uterine cytosol are 1·3, 0·6 and 0·9 respectively (relative to 17β-oestradiol = 100). Other isoflavones are even less active: daidzein exhibits a relative binding affinity of 0·1 and 0·09 for sheep and rat uterine cytosol respectively; biochanin A has an affinity of 0·07 for rat uterine cytosol; and formononetin 0·01 for binding to sheep uterine cytosol (Verdeal and Ryan 1979). The isoflavone metabolites equol, O-desmethylangolensin and angolensin (see below) had relative affinities for sheep uterine cytosol of 0·4, 0·05 and 0·03 respectively (Shutt and Cox 1972).

Based upon the competitive binding to oestrogen receptors in steroid-binding globulins from human breast cancer cells (line MCF-T) the affinities of genistein and formononetin, relative to 17β-oestradiol, are 2 and 0·01 respectively (Martin *et al.* 1978). It seems most likely, as Verdeal and Ryan (1979) have suggested, that transport and metabolic effects are responsible for the apparent discrepancy between the results of the above affinity bioassays and those based upon uterotropic activity. The effects of pure isoflavones in the mouse, rat and sheep are summarized in table 2.

Table 2. Effects of pure isoflavones.[a]

Animal	Compound	Dose	Effect
Mouse	biochanin A	10–40 mg/g diet	uterine hypertrophy
	daidzein	5–15 mg/g diet	uterine hypertrophy
	formononetin	15–40 mg/g diet	uterine hypertrophy
	genistein	5–20 mg/g diet	uterine hypertrophy
	genistein	15 mg/day, diet	infertility, both sexes
	genistein	10 mg injected	displacement of oestradiol from uterine receptors
	genistin	5 mg/day, diet	uterine hypertrophy
	genistin	0·2% diet	infertility, females
	genistin	9–72 mg/day, diet	testes atrophy, depressed growth
Rat	genistein	0·5% diet	testes atrophy, depressed growth
	genistein	0·4 mg, injected	increased protein, phospholipid synthesis in uterus
	genistin	0·5% of diet	testes atrophy, depressed growth
Sheep	biochanin A	1 g, injected	uterine hypertrophy
	formononetin	24 g, injected	uterine hypertrophy
	genestein	1 g, injected	uterine hypertrophy

[a] Full references will be found in Stob (1983), from which this table is taken with permission.

Investigation of the metabolism of isoflavone oestrogens was stimulated by the problem of clover disease in sheep (Bennetts *et al.* 1946). Originally it was assumed that this condition, characterized by a marked loss of fertility, was due to the high levels of isoflavones present in subterranean, and other, clovers. Millington *et al.* (1964) were unable, however, to establish a relationship between the hormonal activity in sheep fed clover and the levels of genistein or biochanin A; a positive relationship was, however, found between the weaker oestrogen, formononetin, and such activity *in vivo*. It is now realized that the reason for this apparently anomalous situation lies in differences in the metabolism of these isoflavones in the digestive tract. Whereas biochanin A and genistein are converted into inactive products, formononetin is metabolized to the isoflavan equol (XVII), and it is this compound in the animal which produces the effect on oestrus (Shutt and Braden 1968). Equol does not, however, appear to be metabolized in the tissues of the sheep (Braden *et al.* 1967). The uterotropic effect of equol is only 10^{-3} that of 17β-oestradiol (Tang and Adams 1980), a potency which is consistent with its relative molar binding affinity to uterine cytosol receptor *in vitro* (Shutt and Cox 1972).

The major pathways which have been elucidated for the metabolism of formononetin (VIII) are shown in figure 4. The primary route, A, involves initial

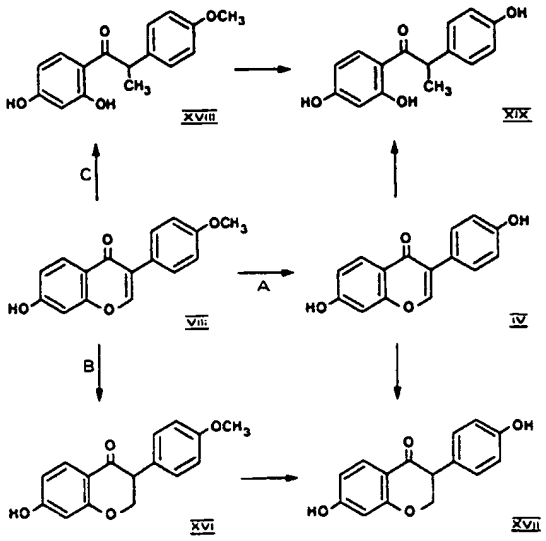


Figure 4. Metabolism of formononetin in the sheep.

demethylation (forming daidzein, IV) and then reduction. A secondary process (B) involves reduction to the 4′-methyl ether of equol (XVI) followed by demethylation. Equol possesses about one half of the affinity for binding to receptor sites of sheep uterine cytosol exhibited by genistein and approximately one quarter of the uterotropic activity of this compound when assayed by intravaginal tetrazolium reduction after oral administration to mice (Shutt and Braden 1968). About 70% of the formononetin ingested by sheep is converted to equol (Shutt *et al.* 1970) and, to a smaller degree, daidzein; in addition, other active metabolites, angolensin (XVIII) and O-desmethylangolensin (XIX) (figure 4, route C) may also be formed (Batterham *et al.* 1971). These compounds are uterotropic in mice and bind to sheep uterine cytosol receptor sites (relative affinities, angolensin 0·03 and O-desmethylangolensin 0·05) (Shutt and Cox 1972). In agreement with the findings of Bickoff *et al.* (1962), referred to above, the oestrogenic activity of the 4′-methyl ether, angolensin, was lower than that of its 4′-desmethyl analogue (Micheli *et al.* 1962).

In marked contrast to the above, biochanin A is metabolized in the sheep via demethylation (to genistein, V) and thence, by ring cleavage (presumably involving the intermediate phenyl-α-methylbenzyl ketone) to the oestrogenically inactive *p*-ethylphenol (XX, figure 5) (Braden *et al.* 1967).

Comparative studies in sheep and cattle revealed the latter to metabolize formononetin more rapidly and also to be more effective in conjugating isoflavones and their metabolites (Braden *et al.* 1971). In sheep the metabolism of biochanin A and genistein in the rumen is initially low but increases significantly over the first few days of grazing on clover and related forages; this is paralleled by a reduction in the hormonal effect of these crops. In marked contrast, the rate of formononetin degradation is not affected by time to any great extent, hence the pasture retains its oestrogenicity

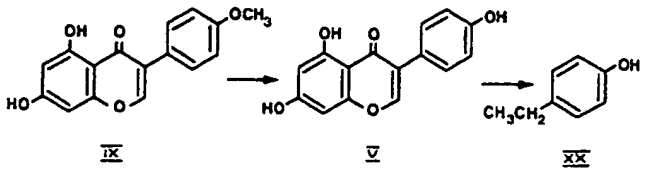


Figure 5. Metabolism of biochanin A in the sheep.

(Lindsay and Francis 1969). Provided that the livestock are removed from such pasture the oestrogenic effects are reversible. However, continued grazing will lead to permanent physiological changes of the reproductive tract (Lindner 1976). Equol has been identified in the urine of goats, rats and hens and in each case was considered to result from dietary isoflavone precursors, rather than being present in the diet *per se*.

Shutt *et al.* (1970) have observed the metabolism of isoflavones in sheep to proceed rapidly; for example, 1 g of biochanin A plus genistein was metabolized in about 90 min. Moreover, the data presented suggested that the initial demethylation (A in figure 4), rather than the reduction, was the rate-limiting step. Equol does not appear to suffer extensive degradation in the rumen, but is readily absorbed therefrom (residence time 1·7 h). There is a suggestion that residence times for isoflavones may be reduced under grazing conditions, a consequence of which may be the less complete metabolism of isoflavones in the rumen, a greater concentration of genistein resulting and/or a decreased production of equol from formononetin (Shutt *et al.* 1970). Consequently the oestrogenic activity, and effect, of such pasture in livestock depends upon the fine balance of isoflavone metabolism *in vivo*.

In contrast to the metabolites of steroidal oestrogens, isoflavones are readily conjugated as glucuronides and excreted. According to Shutt *et al.* (1967) circulating isoflavones are almost exclusively present in the form of biologically inactive glucuronides, although small amounts of the free compounds and their sulpho-conjugates, which can yield the free compounds *in vivo*, may also occur. A similar situation has been observed in man (Axelson *et al.* 1982). The plasma content of dietary isoflavones in sheep following the feeding of red or subterranean clovers was maximal 30 min after feeding and thereafter rapidly declined; the content of equol increased from 4 to 10 μg/100 ml plasma between 30 and 150 min after feeding, whilst the measured conjugated equol in plasma was very much higher (300–400 μg/100 ml) and was largely independent of feeding time (Shutt *et al.* 1967).

Equol was first reported in human urine by Axelson *et al.* (1982). Total daily excretion levels of two male subjects were 10·9 and 35·2 μg, whilst those of four female subjects ranged from 10·7 to 43·3 μg. In most cases $\geqslant$99·8% of the measured equol was excreted as the glucuronide, but in two subjects 5·7 and 9·9% was bound as the sulphoconjugate. Independently Adlercreutz *et al.* (1982) reported that there was no significant difference in the daily urinary excretion of equol by post-menopausal women who were vegetarians (mean 35·8 μg, range 0–113 μg), omnivores (mean 35·8 μg, range 0–102 μg) or suffering from breast cancer (mean 27·2 μg, range 0–74 μg). The maximum mean daily excretion measured was 565 μg over a three-day period, and at such a level the authors considered that a biological effect might result. Subsequently Bannwart *et al.* (1984) identified both daidzein and equol monoglucuronides in the urine of five female subjects. The levels found in four vegetarian subjects (two pre- and two post-menopausal) were much greater than that measured in the single pre-menopausal,

omnivorous subject (daidzein: average 396·6 μg/l, range 96·0–1108 μg/l compared with 21·6 μg/l; equol: average 4207 μg/l, range 1493–9663 μg/l compared with 46·0 μg/l). The variation in the vegetarian subjects was ascribed to differences in the composition of the diet. Apples, cherries, potatoes, garlic, hops and soya products were mentioned as the most probable sources of dietary oestrogenic compounds. A less important source of these compounds was considered to be products obtained from animals which had been fed oestrogen-containing forage. This seems probable, although only limited data is available upon which to base a judgement. According to Lindner (1967) the levels of such isoflavones accumulating in the adipose tissue of sheep (1 μg/g) was too low to present a serious health hazard. The effects of cooking and/or processing would, moreover, seem likely to reduce this figure further.

Recent work has emphasized the importance of soya as a source of dietary isoflavones (Axelson *et al.* 1984). Two healthy subjects were given 40 g of commercial texturized soya in place of meat, daily for 5 days. Urinary excretion of equol was found to increase 100–1000 fold (figure 6) and traces of daidzein glucuronides were also observed. Quantitatively similar results were observed in rats, approximately 100 μg of equol being excreted per gram of soya flour ingested. The figure for soya oil is much less (5 μg/g), indicating that little, if any, isoflavones are removed from soya during processing of the oil (see below). This result is of interest also since Vague *et al.* (1957) have reported cornification of the vaginal epithelium to occur in post-menstrual women following the administration of 100 g corn or olive oil per day for 10 days. The uterotropic effect of soya meal and soya-based rations in laboratory animals and poultry is well documented (Drane *et al.* 1980).

Setchell *et al.* (1984) have recently shown that certain people excreted little or no equol in the urine when fed 40 g of commercial soya protein daily for 5 days. The reasons for this behaviour are unclear, although it appears to be unrelated to the sex of the subject; the authors suggest that the rate of formation of equol was dependent upon dietary-related factors, such as the composition of the intestinal microflora, the intestinal transit time and variability in the redox level of the large intestine. These

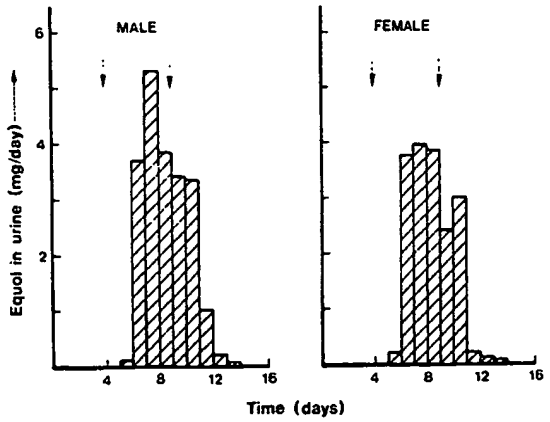


Figure 6. Daily urinary excretion of equol in humans (from Axelson *et al.* 1984). The arrows mark the period over which soya protein, 40 g/day, was fed.

workers also demonstrated that human faecal flora were able to degrade soya-rich broth components (presumably daidzin and daidzein) to equol. As the authors point out, it would be of dubious value to extrapolate the above findings, based upon six subjects, two of whom were obvious non-responders, to the population at large. These results do, however, emphasize the need for further study into the factors affecting phytoestrogen metabolism in man, the metabolic fate of dietary oestrogens in non-responders, and the variation in the rate of phytoestrogen metabolism in larger populations. The latter may in turn lead to the identification of particular 'at-risk' groups within the population at large.

Axelson and Setchell (1981) were unable to determine equol in the urine of germ-free rats fed a commercial, soya-containing ration. Glucosidases capable of converting isoflavone glycosides to the uterotropically active aglucones have been identified in man, as have enzymes which have been shown to carry out the conversion of daidzin to equol (Axelson *et al.* 1984). Although no evidence has yet been presented it would be expected that genistin→genistein→*p*-ethylphenol would represent a similar (but *de*toxifying) metabolic pathway in man.

The improvement of methods for the detection and quantification of isoflavones and their metabolites in plant material and biological samples has been of great importance in the development of an understanding of their chemical and biological properties. Such analysis has been effected by a variety of techniques, including paper chromatography (Markham 1975), thin layer chromatography (Beck 1964), gas chromatography (Naim *et al.* 1974), high-performance liquid chromatography, spectrophotometry, fluorimetry and immunoassay. Gas chromatographic methods, either alone or linked to mass spectrometry, have involved the prior derivatization of the molecules by converting free —OH groups to trimethylsilyl ethers or trifluoroacetyl esters (Naim *et al.* 1973). Gas chromatography-mass spectrometry procedures such as single ion monitoring have allowed very low levels of isoflavone metabolites to be measured (Bannwart *et al.* 1984, Axelson *et al.* 1984), and using conventional gas chromatography procedures the former authors have demonstrated a detection limit of 1 μg equol/24 h urine sample. With such low levels, the isolation, extraction and concentration of the compound(s) of interest from the bulk sample is of paramount importance. The use of reversed-phase silica for preliminary clean-up has proved especially useful for the extraction of isoflavones and equol (and their conjugates) from urine, following which enzyme hydrolysis and ion exchange clean-up processes are employed (Axelson *et al.* 1984, Bannwart *et al.* 1984). Use of DEAE Sephadex (base form) enables a degree of separation between mono- and diphenolic species to be effected (Axelson *et al.* 1982).

The advantage of high-performance liquid chromatography techniques is that the samples can be examined without the need for derivatization; under such conditions both free compounds and conjugates may be analysed directly. Following the original report of Kallela and Saastamoinen (1978), a number of techniques have been described, which almost invariably use reversed-phase systems. Methods developed for the analysis of isoflavones in clover and other fodder crops usually rely upon the facile hydrolysis of the glucosides during plant maceration and extraction such that the isoflavone aglucones are separated and quantified. Petterson and Kiessling (1984) and Sachse (1984) both include chemical hydrolysis prior to sample analysis. Free isoflavones and glucosides are readily determined in soya by high-performance liquid chromatography and, of the methods described, the present authors favour that of Eldridge (1982a) in which all of the likely soya isoflavones are separated, an internal

standard is included and no problems of co-eluting impurities are encountered. The latter severely limits the usefulness of a semi-preparative method for the isolation of daidzin and genistin, reducing the loading capacity to an extent that conventional chromatography (using Sephadex LH20) was of comparable efficiency (Farmakalidis and Murphy 1984a).

The analysis of soyabean (meal) and fractions have almost invariably revealed the presence of daidzin, daidzein, genistin, genistein, glycitin-7β-glucoside and glycitein, the latter two being uterotropically inactive and for this reason not included in table 3. Small amounts of formononetin were also claimed to be present by Shemesh *et al.* (unpublished, cited in Lindner 1976), but details of the method were not given; in the absence of any independent confirmation and bearing in mind the obvious differences between the results of these workers and others (table 3) for the levels of the other isoflavones, this report should be treated with caution. It is generally held that the major proportion of soyabean isoflavones are present as glucosides (table 3), but as has been indicated these are readily degraded by intestinal bacteria prior to metabolism, conjugation and excretion. Bickoff *et al.* (1962) have reported that 8 mg of genistein (or 10 mg of daidzein) was the minimum dose needed to induce a hormonal response in mice; hence the oestrogenic effect of soyabean meal and soyabean-containing commercial rations on poultry and laboratory animals is readily understood, especially when it is further realized that biologically significant levels of coumestrol and its methyl ethers may also be present.

Much research on the isoflavones of pasture and forage crops has demonstrated that many factors (e.g. the physiological age of the plant, its genetic origin, climatic and environmental factors associated with growth) can affect the ultimate content of these compounds in the plant (Bickoff 1968, Rossiter and Beck 1967), and more recent work has shown these factors also to be important in soya. However, additional consideration must be taken of the effect of subsequent processing, especially as it relates to human food ingredients.

Eldridge and Kwolek (1983) have shown that the defatting of full-fat soya does not remove isoflavones or their glucosides, contrary to the earlier claim of Booth *et al.* (1960). Support for this later finding comes from the work of Axelson *et al.* (1984) referred to above. Analysis of soyabean hull (8% by weight), hypocotyl (2%) and cotyledon (90%) fractions revealed isoflavone contents of 10–20 mg/100 g, 1405–1750 mg/100 g and 319–808 mg/100 g respectively. It should be noted that coumestrol is concentrated primarily in the hull and testa portions (Lookhart 1979). Daidzin and glycitin account for more than 95% of the total isoflavone content of the hypocotyl, whereas in the cotyledon the latter is almost absent and genistin predominates. Eldridge (1982b) found that soya protein concentrate (containing 70% protein) prepared by aqueous leaching contained higher levels of isoflavones (247 and 317 mg/100 g) than were present when an aqueous alcohol process was used (16 and 43 mg/100 g). Soya protein isolates, containing 90% protein, although obtained by a variety of unspecified procedures, contained similar isoflavone contents (103–145 mg/100 g), most of which was genistin and genistein. Combined levels of daidzin and daidzein, yielding equol on metabolism, ranged between 24 and 51 mg/100 g. Seo and Morr (1984) found a commercial protein isolate to contain 96 mg isoflavones/100 g. Whilst in general agreement with these findings, Murphy *et al.* (1982) observed the level of isoflavone glucosides in soyabeans to decrease substantially on germination, during protein isolation or when calcium-precipitated tofu was prepared. There appeared, however, to be no corresponding increase in the free forms of these isoflavones. According to György *et al.* (1964)

Table 3. Oestrogenic isoflavone content of soya and its products.

Sample	Daidzin (mg/100 g)	Daidzein (mg/100 g)	Genistin (mg/100 g)	Genistein (mg/100 g)	Formononetin (mg/100 g)	Reference
Soyabean meal	62	48	127	40		Eldridge (1982a)
Soyabean meal	11·7, 0	0, 2·2	74·7, 102·4	4·0, 2·4		Murphy (1982)
Soyabean meal	56·7, 56·1	4·9, 14·5	65·5, 81·3	9·7, 18·7		Pettersson and Kiessling (1984)
Soyabean meal	42	17·8	151	108		Pratt and Birac (1979)
Soyabean flakes	59·6 ± 8	5·6 ± 0·7	215 ± 9	6·7 ± 8		Seo and Morr (1984)
Soyabean flour	48–77	8–48	58–154	4–46		Eldridge (1982)
Soyabean cake		30 ± 5		18·6 ± 2·7	4·3 ± 2	Shemesh *et al.* (in Lindner 1976)
Soyabean flakes	114	2·5	188·5	4·4		Eldridge and Kwolek (1983)
Soya-based animal ration	7		42–45	7		Murphy *et al.* (1982)

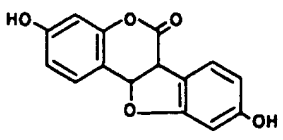
daidzin and genistin are hydrolyzed by *Rhizopus oryzae* during the fermentation of soyabeans to produce tempeh. Defatted soya flakes contained 287 mg isoflavones/100 g (Seo and Morr 1984) and this was decreased by various protein isolation procedures to 203 mg/100 g (acid precipitation), 53 mg/100 g (dialysis), 8·3 mg/100 g (ion exchange) and 6·1 mg/100 g (activated charcoal treatment). A commercial sample of soya protein hydrolysate contained genistein and daidzein contents of 54 and 15·2 mg/100 g, respectively; animal rations containing soya hydrolysates were also observed to possess very low levels of isoflavones (Murphy 1982). Germinated bengal gram (*Cicer arientanum*) was found to contain biochanin A and formononetin at levels of 71 and 77 mg/100 g (Dziedzic and Dick 1982) and 98·6 mg and 44·1 mg/100 g (Sharma 1979a), respectively. The latter worker also identified daidzein (5·1 mg/100 g).

Bartholomew and Ryan (1980) found daidzein, genistein, formononetin and biochanin A all to be non-mutagenic when screened using the *Salmonella*/mammalian microsome assay, the behaviour of the first two compounds being in agreement with the findings of Sugimura *et al.* (1977), and confirmed by Murphy and Glatz (in Murphy 1982).

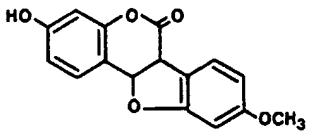
Isoflavone aglucones have been shown to be responsible in part for the antioxidant activity of soyabeans and their products (György *et al.* 1964, Pratt and Birac 1979, Pratt *et al.* 1981). These compounds also contribute to the astringent and bitter tastes of defatted soyabean (How and Morr 1982) and soy protein products (Huang *et al.* 1981). Soyabean isoflavones possess marked antifungal activity, whereas the glucosides are almost without action (Naim *et al.* 1974). Sharma (1979b) has demonstrated that biochanin A, formononetin and pratensein possess hypolipidaemic activity in the albino rat, but daidzein (and genistein (Ollis 1962)) was inactive. It was considered that this, at least in part, explained the hypocholesterolaemic activity of the black gram and navy bean (Saraswati Devi and Kurup 1972, Hellendoom 1976).

Coumestans

Coumestans possess structures exhibiting close similarity to those of isoflavones to which they are biosynthetically related. A relatively large number of these compounds have been isolated from plants (Wong 1975), but only a few have been shown to possess uterotropic activity. For example, Verdeal and Ryan (1979) list eight coumestans which have been identified in alfalfa, only two of which possess such activity. These compounds, coumestrol (7,12-dihydroxycoumestan, XXI) and 4′-methoxycoumestrol (7-hydroxy-12-methoxycoumestan, XXII) (figure 7) are the most common of this class of oestrogen and have been reported in alfalfa, ladino clover and other fodder crops where their presence is associated with widespread problems of animal performance (Stob 1983). According to Hanson *et al.* (1965), over 90% of the oestrogenic activity of potent dehydrated alfalfa samples was due to its coumestrol content and Lookhart



XXI



XXII

Figure 7. Structure of coumestans.

(1980) has found serious oestrogenic effects to result from feeding cattle haylage containing 37 mg coumestrol/kg. Bickoff *et al.* (1960) and others (Micheli *et al.* 1962) have investigated the effect of structural changes in the coumestan molecule on its hormonal activity. Phenolic groups in the 7,12 positions were important; thus the 7-methyl ether and 12-methyl ether (XXII) possessed only 54% and 15%, respectively, of the uterotropic activity of coumestrol itself when administered orally to mice. 7,12-Diacetoxycoumestrol was almost as active as the parent compound when administered in the same manner, presumably reflecting the lability of the acetoxy groupings *in vivo*.

As may be seen from figure 1, the uterotropic potency of coumestrol in the mouse is greater than that of the isoflavones and, as with the latter, variation occurs according to species and means of administration. Braden *et al.* (1967) found coumestrol (administered intraruminally) to be 15 times more active than the most potent isoflavone and it is even more potent when injected intramuscularly. In the mouse, coumestrol is 35 times more active (and its diacetate 24 times more active) than genistein, but still possesses less than 0·03% of the activity of diethylstilboestrol (Bickoff *et al.* 1962). The coumestans, like the isoflavones, bind competitively to mammalian oestrogen receptor sites and are more active when assayed in this manner. The relative binding efficiency (that of 17β-oestradiol = 100) of coumestrol has been reported as 1·4 (rabbit uterine cytosol, Shemesh *et al.* 1972), 4·9 (sheep uterine cytosol, Shutt and Cox 1972), 4·9 (rat uterine cytosol, Verdeal *et al.* 1980) and 19·7 (calf uterine cytosol, Lee *et al.* 1977). When tested in human cancer cell preparations the relative affinity of coumestrol was measured as 9·8 (Martin *et al.* 1978). According to Fredericks *et al.* (1981) coumestrol may exert its effect on fertility *in vivo* by inhibiting follicle stimulating hormone. Little is known about the metabolism of coumestrol; Kelly (1972) has found the compound to be rendered less active in sheep over a period of 7–14 days. Whilst this might be due to the formation of less active metabolites, the chemical nature of which is obscure, more recent work suggests an alternative explanation. Coumestrol is conjugated *in vivo*, but to a rather lower extent than the isoflavones. Thus Kelly and Lindsay (1978) found between 20% and 40% of the total coumestrol in sheep's plasma to be present in the free form (compared to less than 10% in the case of the isoflavones) (Shutt *et al.* 1967). Significantly the concentration of free coumestrol in sheep's plasma remained constant over 16 days, during which time the animals became biologically less sensitive to the oestrogenic effects of this compound. The loss of sensitivity, moreover, appeared to be related to the amount of dietary coumestan and the period of exposure. Further work is needed to clarify the factors underlying these interesting observations. The biological effects of administering coumestrol to animals is shown in table 4.

Coumestrol has been found in a range of plant products commonly consumed by man (table 5). The highest levels were noted in sprouts of alfalfa and, especially, soyabean (Knuckles *et al.* 1976b). Legume sprouts and shoots have in recent years been consumed in increasing amounts by certain sections of the population of the UK and other western countries. It would seem prudent to conduct a more detailed study of the coumestrol (and isoflavone) contents of these materials using modern analytical methods. In the aforementioned work, Knuckles *et al.* used paper chromatography allied to fluorimetric detection and quantification (Knuckles *et al.* 1976a); at the present time, however, the best method of analysis would appear to be high-performance liquid chromatography (Lookhart *et al.* 1978, 1980) using ultraviolet or fluorimetric detection. By judicious choice of mobile and stationary phases it is also possible to monitor isoflavones and coumestrol simultaneously (Pettersson and Kiessling 1984).

Table 4. Effects of pure coumestans and zearalenone.[a]

Animal	Compound	Dose	Effect
Mouse	coumestrol	100–500 μg/g diet	uterine hypertrophy
	coumestrol	500 μg/g diet	antigonadotropic
Rat	coumestrol	1 mg injected, 5 days neonatally	persistent oestrus syndrome
	coumestrol diacetate	125 μg injected	increased protein and phospholipid synthesis in uterus
Sheep	coumestrol	12 mg injected, 1·4 g intraruminally	uterine hypertrophy
Mouse	zearalenone	10 μg/g diet	uterine hypertrophy
		20 μg injected	uterine hypertrophy
Rat	zearalenone	1 mg, oral	uterine hypertrophy
		600 μg topical to skin	uterine hypertrophy
Swine	zearalenone	1–50 mg daily, oral	hypertrophy vulva, vagina, uterus and mammae; metaplasia of cervical epithelial cells
		100 μg/g diet	infertility
		25–100 μg/g diet	infertility, nymphomania, pseudopregnancy, reduced litter size, smaller pigs, malformations, juvenile hyperoestrogenism, probable fetal resorption
Chicken	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Turkey	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Monkey	zearalenone	14 or 56 μg/kg injected	stimulation, LH[b] surge
		14 μg/kg injected	serum LH depression
		400 μg daily, orally for 4 days	serum LH depression

[a] Full references will be found in Stob (1983), from which this table is taken with permission.
[b] LH = luteinizing hormone.

Table 5. Coumestrol content of plant products.[a]

Product	Coumestrol content (μg/100 g dry weight)
Alfalfa sprouts (fresh)	500
Soyabean sprouts (fresh)	7110
Soyabeans (dry)	120
Defatted soyabean meal (dry)	40
Soyabean concentrate	20
Soyabean isolate	60
Frozen green beans	100
Frozen snow beans	60
Frozen green peas	40
Frozen Brussels sprouts	40
Dried red beans	40
Dried split peas	30
Frozen spinach leaf	10

[a] Data from Knuckles *et al.* (1976) with permission.

The coumestrol content of plant material has been observed to vary with a variety of factors (Bickoff *et al.* 1969). For example, Hanson *et al.* (1965) have shown that of alfalfa to be affected, to various degrees, by variety, stage of growth, cutting, the year and location and, to a significant degree, by the presence of disease. Coumestrol has been observed to accumulate in alfalfa and other legumes following insect (Loper 1968) or fungal attack (Loper 1968, Loper and Hanson 1964, Stuthman *et al.* 1966, Loper *et al.* 1967). According to Sherwood *et al.* (1970) coumestrol was not translocated from the infected area to other parts of the plant. Whereas coumestrol in undamaged, non-infected plants was metabolized via the isoflavone pathway (Grisebach and Barz 1963, 1964), the origins of the coumestrol biosynthesized as a result of such insect or fungal damage is unknown.

Concern over the presence of coumestans in alfalfa and ladino clover has resulted from the reduced reproductive performance of animals maintained on such fodder (Hanson *et al.* 1965, Bickoff *et al.* 1969) and both breeding programmes and improved husbandry practices have been initiated to reduce the extent of the problem. Of the latter, treatment with agrochemicals can minimize the pest and fungal attack which results in accumulation of coumestrols and other plant phenolics; moreover, the intake of coumestrol by animals can also be reduced by the feeding of immature plants in which the coumestrol content is known to be lower than in the mature plant. In the absence of any information concerning the amount, if any, of coumestans which enter the human body indirectly via the residues in animal products and milk obtained from livestock grazing on oestrogenic pasture, concern over the intake of these compounds by man is centred mainly upon their presence in common food plants (table 4), vegetable protein and 'health' products.

Leaf protein concentrate has been suggested as a source of protein for humans, and methods have been described for its preparation from alfalfa (Kohler *et al.* 1968, Edwards *et al.* 1975). The effect of such processing on the coumestrol content has been examined by Knuckles *et al.* (1976b). Relatively little of the original coumestan content of the alfalfa (11–118 mg/kg) was removed in the solubles during the early stages of the processing. Protein concentrates possessing 9–14 mg coumestrol/kg were obtained by commercial-type processing in which heat coagulation and washing was carried out under acid conditions (pH 4·5–6·5), whereas if the medium was kept alkaline (pH 8·5–9·5) the coumestrol content was much lower (3 mg/kg) due to the greater solubility of the oestrogen under these conditions. Diafiltered alfalfa leaf protein concentrate possessed a coumestrol content of only 0·4 mg/kg (measured as freeze-dried powder). Since the coumestrol content of diseased or damaged alfalfa leaves may exceed 1000 mg/kg, i.e. 10–100 times that of undamaged tissue, it is clearly important that the quality of the materials selected for processing be maintained as high as possible.

Alfalfa and other leguminous products have been widely marketed in recent years as health foods, tonics and supplements. Recently, Elakovich and Hampton (1984) have analysed commercial alfalfa tablets and found these to contain 20–194 μg coumestrol/g, equivalent on a daily dosage basis to 1–2 mg of coumestrol. The effect of long-term exposure to such levels (together with that of any isoflavone oestrogens which may also be present) cannot yet be ascertained. However, this work clearly points to the desirability of monitoring the contents of physiologically active substances in health products since the 'recommended' doses (if stated) are frequently exceeded and the products may not be covered by the same legislative controls as foods and feeding-stuffs.

Coumestrol has been observed to possess tumour-promoting activity similar to that of 17β-oestradiol and diethylstilboestrol for dimethylbenzanthracene-induced rat mammary tumours (Verdeal *et al.* 1980). However, Bartholomew and Ryan (1980) have reported this compound to be non-mutagenic in the Ames test. Both coumestrol and its 4'-methyl ether had been shown to possess weak antifungal activity (Van Etten 1976).

Resorcylic acid lactones

Unlike the previous two groups of plant oestrogens, the resorcylic acid lactones are not intrinsic components of food plants but are secondary mould metabolites of fungal species, principally *Fusarium*, e.g. *F. roseum* var. *graminearum* (*Gibberella zeae*) which are common field organisms which also proliferate in poorly stored grains, oil seeds and hay (Caldwell *et al.* 1970, Eugenio *et al.* 1970, Sherwood and Peberdy 1972, Abbas *et al.* 1984). There have been a number of detailed reviews on the chemistry (Shipchandler 1975), production and biological activity (Mirocha *et al.* 1971, 1977, Mirocha and Christensen 1974, Pathre and Mirocha 1976, Hidy *et al.* 1977, Betina 1984) of these compounds and a comprehensive coverage of these and other aspects of *Fusarium* moulds is now available (Moss and Smith 1984). The economic losses associated with the feeding, especially to swine and cattle, of rations containing such mould-damaged produce have rightly meant that emphasis is primarily placed on the effects of such compounds on livestock, rather than on humans. However, since there is, at least in principle, the possibility of these compounds being carried over into humans via the consumption of animal products, and as many grain and cereal products are now formulated directly for human consumption, it is appropriate to consider the levels of such compounds likely to enter the human body and, thereby, assess the likely risk from such compounds.

The most common oestrogen of this group is zearalenone (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl) β-resorcylic acid lactone, XXIII). The reduced compound, zearalanol (XXVI, figure 8), has been marketed as a growth promoter in sheep and bovines. The main metabolites of zearalenone are the epimeric β- and α-zearalenols (XXV and XXIV). Other compounds have been identified (Verdeal and Ryan 1979), but in general little is known about their biological activity. Zearalenone is usually described as a mycotoxin (F-2 toxin) but some reviewers consider this as inappropriate (Stob 1983). There have been a number of cases reported where the feeding of *Fusarium*-infected rations have caused death, abortion and other serious physiological disorders in livestock and poultry, and in some cases these were attributed to the presence of zearalenone. It is possible that other, more toxic, substances were also present, since such moulds normally produce a number of mycotoxins simultaneously; these may include trichothecenes, such as T-2 toxin, deoxynivalenol and diacetoxyscirpenol. As Stob (1983) has stated, the involvement of zearalenone in some of the more distressing symptoms associated with the feeding of *Fusarium*-infected rations should be treated with circumspection and the role of zearalenone itself should be demonstrated in controlled feeding trials, where such additional compounds can be excluded. The observed LD_{50} of zearalenone is certainly far removed from those of other mycotoxins, being 5, 10 and 20 g/kg in female guinea pigs, rats and mice respectively. For these and other reasons, Stob (1983) has suggested the terms 'mycoestrogen', 'fungal oestrogen' or 'oestrogenic metabolite' as being more appropriate.

Zearalenone, zearalenol and zearalanol have been found to bind to mammalian

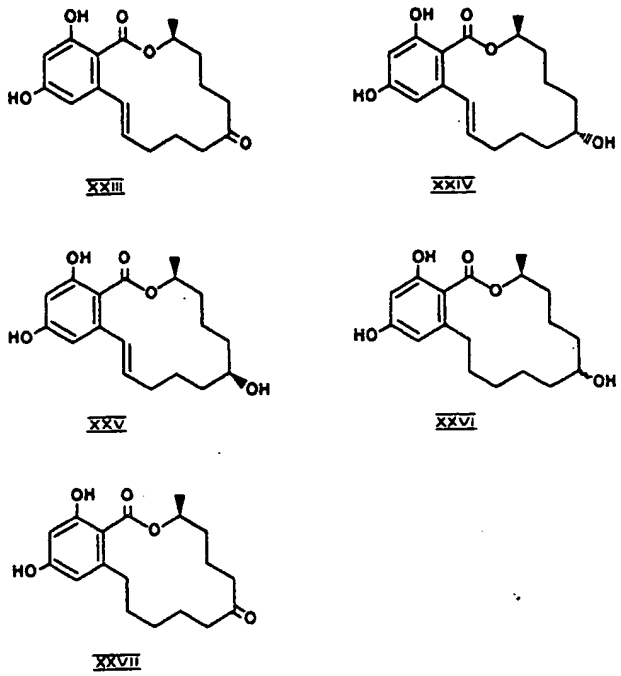


Figure 8.

oestrogen receptor sites. Kiang *et al.* (1978) showed these to bind to uterine cytosol and nuclear receptors in the order *cis*-zearalenone (not naturally occurring) > *trans*-zearalenone > zearalenol (stereochemistry unspecified) > zearalanol. All four compounds almost completely inhibited the binding of 17β-oestradiol at a ratio of 100:1. Katzenellenbogen *et al.* (1979) found α-zearalenol to be more active than either β-zearalenol or zearalanone when measured by competitive or direct binding assays using rat uterine cytosol receptors. The former compound was observed to possess 13·6% and 15% of the effect of 17β-oestradiol upon competitive and direct binding analysis, respectively.

It has been suggested (Ueno and Tashiro 1981) that the oestrogenic effect of zearalenone is due to its metabolism to zearalenol, and this suggestion has been supported by more recent work (Sheehan *et al.* 1984). Despite the structural dissimilarity between zearalenone and 17β-oestradiol, as Duax *et al.* (1984) have pointed out, there is considerable similarity between their respective hydrophobic bulk. Zearalenone binds to rat hepatic cytosol oestrogen receptors (Powell-Jones *et al.* 1981) as well as to those of rat uterus, with which it has been found to bind more strongly than the isoflavones but less strongly than coumestrol (Verdeal *et al.* 1980). Radio-labelled zearalenone, injected intravenously into mice, was found to be bound to oestrogen target organs, e.g. uterus, intestinal testicular cells and ovarian follicles (Appelgren *et al.* 1982). Studies by Martin *et al.* (1978) showed that zearalenone was less potent than

either isoflavones or coumestrol when assayed by competitive binding to human breast cancer cell oestrogen receptors. The uterotropic activity of zearalenone has been demonstrated by various workers (Stob 1983)—for example, when administered by mouth it was 10^3 times less active in the mouse than was diethylstilboestrol (figure 9). By subcutaneous injection in the same species, the compound was 500 times less active than 17β-oestradiol (Katzenellenbogen *et al.* 1979). Mirocha *et al.* (1978) have shown *cis*-zearalenone to possess stronger uterotropic activity than the natural *trans*-isomer; *cis*- and *trans*-zearalenols were found to be of comparable activity by the same workers.

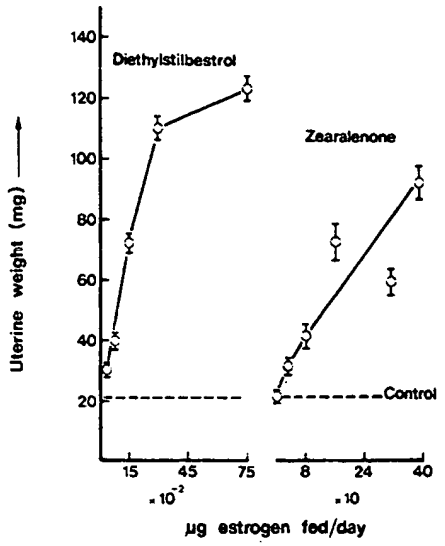


Figure 9. Relative uterotropic activity of orally administered diethylstilboestrol and zearalenone (after Stob 1983).

The biological activity of zearalenone in animals is shown in table 4. Data on the oestrogenic potency of zearalenone in rats has been obtained by Kumagai and Shimizu (1982); by uterotropic assay this compound possessed less than 0·1% of the activity of 17β-oestradiol, a figure in agreement with that resulting from estimation of vaginal cornification following systemic administration. In contrast, the vaginal cornification bioassay indicated that zearalenone possessed about 1% of the activity of 17β-oestradiol when administered locally. Furthermore, neonatal exposure to zearalenone produced anovulatory sterility in the rat, the potency being 10% that of 17β-oestradiol. There is considerable evidence that pigs are especially sensitive to zearalenone (Mirocha *et al.* 1974, Chang *et al.* 1979) with hormonal effects resulting from as little as 1–5 mg/kg diet. According to Chang *et al.* (1979), the inclusion of 25–100 mg zearalenone/kg in the ration of sows led to multiple reproductive deficiencies, including infertility, reduced litter size and weight and hyperoestrogenism. Zearalenone and zearalanol have been shown to increase weight gain when implanted subcutaneously in sheep (Hidy *et al.* 1977) and subsequent study demonstrated the latter to be especially

effective in steers and heifers (Willemart and Bouffault 1983). Further work also resulted in synthetic zearalanol implants being produced commercially.

In primates, orally administered zearalenone was 80 or 160 times less active than 17β-oestradiol or diethylstilboestrol, respectively, in inhibiting synthesis and release of gonadotropins (luteinizing hormones) from the anterior pituitary (Hobson *et al.* 1977), but the effect is much greater if the compounds are administered by subcutaneous injection and all three compounds are then of comparable effectiveness. In humans, 75–100 mg of zearalenone/day have been reported to be effective in the treatment of post-menopausal syndrome in women (Utian 1973, Hidy and Baldwin 1976b) and, according to Hidy *et al.* (1977), the clinically effective daily dose of zearalanol for such purposes is 50–75 mg, a practical result which was in agreement with that inferred from animal experiments. Both zearalanol and zearalenone are effective as oral contraceptive agents in humans (Hidy *et al.* 1976a).

According to unpublished work cited by Hidy *et al.* (1977), the major metabolite of orally administered zearalenone in the sheep was β-zearalenol (XXV) which was shown to possess only 25% of the oestrogenic activity of the parent compound. Ueno and Tashiro (1981) observed only small amounts of this metabolite in rat faeces, the main product being the epimer, α-zearalenol (XXIV), which was three times more uterotropically active than zearalenone and also bound more strongly to mammalian uterine cytosol receptor. α-Zearalenol may be present in both the free and conjugated (β-glucuronate) forms (Kiessling and Pettersson 1978, Olsen *et al.* 1981) and conjugation of zearalenone itself may also represent a significant detoxification process (Kiessling and Pettersson 1978, Olsen *et al.* 1981) in the rat. Rumen-microbes have been observed to metabolize zearalenone into α- (mainly) and β-zearalenols and it has been suggested that an additional explanation of the oestrogenic effect of zearalenone-zearalenol metabolism is the interruption of normal steroid metabolism via the necessary involvement of hydroxysteroid dehydrogenase (Kiessling and Pettersson 1978, Olsen *et al.* 1981). As has been mentioned earlier, zearalanol has been used to improve weight gain of livestock. The main metabolite of this compound in a wide range of species, including man, is zearalanone (XXVII); both compounds have been found in the free and bound forms (Hidy *et al.* 1977).

According to Dixon and Russell (1983), when four cattle were implanted with 36 mg of zearalanol, mean maximum urine levels were 13·5 μg/l (22 days after implantation) and declined to 2·9 μg/l by day 69. Two experiments were conducted with sheep, who received 12 mg zeralanol implanted into the base of the ear. Mean maximum urine levels were reached after 35 days (45 μg/l) and 56 days (90 μg/l) in the two experiments, and thereafter declined to 26 μg/l (day 42) and 11·7 μg/l (day 70), respectively. Ruddick *et al.* (1976) reported zearalenone to be teratogenic although more recent work (Davis *et al.* 1977, Wardell *et al.* 1982) could not confirm this.

Numerous methods have been described for the analysis of zearalenone (Gilbert 1984), including thin layer and paper chromatography (with colourimetric or ultraviolet detection) (Caldwell *et al.* 1970), gas chromatography (utilizing the trimethylsilyl- or pentafluoropropionate derivatives) (Steele *et al.* 1976, Holder *et al.* 1977), gas chromatography-mass spectrometry (Mirocha *et al.* 1974, Scott *et al.* 1978) and high-performance liquid chromatography (Scott *et al.* 1978, Cohen and Lapointe 1980). Thin layer and high-performance liquid chromatography have also been used to separate zearalenone and its metabolites in biological samples (Kiessling *et al.* 1984, Ueno and Tashiro 1981). The detection limit for zearalenone using high-performance liquid chromatography, with Spherisorb 5 μm column and fluorescence detection, was

5 μg/kg in corn flakes (although a second high-performance liquid chromatography column was needed to remove an interfering compound in other corn products) and 10 μg/kg in corn (Scott *et al.* 1978, Ware and Thorpe 1978, respectively). For purposes of routine screening, simpler techniques using thin layer chromatography have been developed. With Fast Violet B as spray reagent, detection limits of 20 μg/kg (Scott *et al.* 1978) and 80 μg/kg (Swanson *et al.* 1984) have been reported for zearalenone in corn and corn-based foods. The latter workers also considered the method amenable for the qualitative, but not quantitative, screening of zearalenol (detection limit 200 μg/kg). Immunological techniques have been developed for the detection and quantification of zearalanol (Dixon 1980, Dixon and Russell 1983, Thouvenot and Morfin 1983) but since related compounds, such as zearalanone and zearalenone, may possess significant cross-reactivity towards the antiserum, a preliminary separation with high-performance liquid chromatography has been recommended (Jansen *et al.* 1984).

The extent of the contamination of grain crops with *Fusarium* species may be considerable. In 1972, 38 out of 223 corn samples from areas in the USA where such contamination was suspected or expected were found to contain zearalenone, the levels ranging from 100 to 5000 μg/kg (Eppley *et al.* 1974). A similar study the following year revealed zearalenone levels of 38–294 μg/kg in 19 out of a total of 315 marketable corn samples (Stoloff *et al.* 1976). There was clear evidence of localized regional occurrence with 10% of the samples from the Corn Belt (17 out of 169) being affected. The same workers also measured zearalenone contents of 97–10 400 μg/kg in 57 samples of obviously damaged corn. The results of other surveys of wheat, grain sorghum, soyabeans and corn have been summarized by Bennett and Shotwell (1979). Zearalenone has been detected in six samples of Mexican corn intended for human consumption, but the levels were not quoted (Mirocha *et al.* 1972). Of 293 samples of the 1982 Australian maize crop recently examined (Blaney *et al.* 1984), 85% contained zearalenol; the mean concentration was 170 μg/kg but four samples possessed in excess of 1000 μg/kg. Côté *et al.* (1984) found 40 out of 342 feed samples, obtained in 1981 from the area around Illinois and suspected of causing or contributing to animal health problems, to contain zearalenone. Levels ranged from 100 to 8000 μg/kg, with a mean of 660 μg/kg. In Canada, problems associated with *Fusarium* infection of corn and other crops would seem to occur predominantly in Ontario (Andrews *et al.* 1981). Analysis of suspected samples over the period 1972–1977 revealed some 10% (214 out of 2022) to possess zearalenone, levels ranging from 10 to 141 000 μg/kg, the mean being 3850 μg/kg. Zearalenone, deoxynivalenol and, apparently for the first time, aflatoxin B_1, have recently been identified in commercial wheat samples from the mid-western USA. Of a total of 33 samples examined, zearalenone was present in trace amounts in two samples and, in another three, at levels of 35, 90 and 115 μg/kg (Hagler *et al.* 1984).

There is some disagreement over the effectiveness of chemical treatments for detoxification of zearalenone-contaminated grain. An American patent (Tamas and Wöller 1977) describes either 3–6% aqueous hydrogen peroxide or ammonium hydroxide as effective, but the removal of zearalenone was not quantified. However, unpublished work, referred to by Bennett and Shotwell (1979), found the ammoniation process used for removal of aflatoxins to have no effect on zearalenone levels. More recently, Kallela and Saastamoinen (1981) have shown the farm grain preservative 'Gasol' to have a beneficial effect in reducing the levels of zearalenone in stored grains.

A considerable amount of the world grain crops is used as human food sources, either directly or after processing. In many parts of the world such use represents the

major part of the crops' utilization. There have been a number of reports of zearalenone being found in southern African foods, drinks and raw materials. Thus levels of 100–800 μg/kg were measured in corn used for the brewing of Zambian beer (Lovelace and Nyathi 1977) with an average of 920 μg/kg (maximum 4600 μg/kg) being found in such beers and 800–4000 μg/kg in the corn malt used in the brewing process. Of 55 samples of sour drinks, porridges and beers from Swaziland, six were found to contain zearalenone (referred to in Bennett and Shotwell 1979) at levels between 800 and 5300 μg/kg. Of local beers from Lesotho, 12% of the 140 samples examined also contained this oestrogen (300–2000 μg/kg). Rather lower levels were found in Lesotho beer by Martin and Gilman (1976) (approximately 50 μg/kg) and samples of maize porridge, sorghum malt were also found to be contaminated (Martin 1974). MacDonald and Raemakers (1974) found zearalenone in South African maize samples. Together with zearalenone, the presence of other, more toxic, metabolites may be expected (Bennett and Shotwell 1979) and although the climate in southern Africa might be expected not to be such as to facilitate such mould growth as might occur in other parts of the world, Marasas *et al.* (1977) have found strains of *F. graminearum* in southern Africa capable of producing deoxynivalenol, and possibly other mycotoxins.

According to Stoloff and Dalrymple (1977), zearalenone was not detected in the primary or by-products from dry milling operations. Bennett *et al.* (1976, 1978) have examined the effects of processing on naturally contaminated corn. Wet milling was found to concentrate the oestrogen in the gluten fraction with lesser amounts being found in the milling solubles, fibre and germ respectively. The starch fraction was free of zearalenone. Dry milling led to a two- to three-fold concentration of the zearalenone in the germ. Both milling processes led to a concentration of the zearalenone into fractions used as animal feedingstuffs.

Scott *et al.* (1978) have examined various corn products for zearalenone using both high-performance liquid chromatography and gas chromatography. Largest amounts were found in a sample of cornmeal (26 μg/kg), although two other samples contained no detectable amounts. Frozen corn contained 2 μg/kg, corn chips 0 and 2 μg/kg, popcorn 0 and 7 μg/kg and three samples of cornflakes 0, 0·4 and 14 μg/kg respectively. The carry-over of zearalenone in cattle consuming naturally infected wheat rations has been studied by Shreeve *et al.* (1979). Concentrates (385–1925 μg zearalenone/kg) were fed to two cows for 7 weeks. No zearalenone (detection limit 4 μg/kg) residues were detected in muscle, kidney, liver, serum, milk or urine. The result should be interpreted with some caution bearing in mind the number of animals used and the inability of the analytical method used to detect zearalenone metabolites. The study also revealed apparent indications of interactions between dietary fungal metabolites which would warrant further examination. Mirocha (1981) has detected α- and β-zearalenol (16–76 μg/kg) in the milk of a cow following the oral dosage of [^{3}H]zearalenone. Palyusik *et al.* (1980) have described the results of feeding two lactating sows a diet containing pure zearalenone (40 mg/kg). In addition to various physiological effects attributable to the oestrogenic effect of this compound, analysis of the milk from these animals showed mainly β-zearalenol (> 80% of original toxin) and α-zearalenol (~15%) with only traces (0·5–1·3%) of unchanged zearalenone. The highest concentration of zearalenol found in milk was 0·79 p.p.m. The authors reported that the metabolites could be detected in the milk samples within 2 days of feeding the zearalenone and were still present 5 days after it had been removed from the diet.

Calculations quoted by Lovelace and Nyathi (1977) give possible daily intakes of zearalenone of 450 μg and 170 μg for rural farmers in Southern Province and

inhabitants of Lusaka, respectively. The figure for certain individuals is certainly much higher. Marasas *et al.* (1979), on the basis of animal data, considered that 500 μg/kg was a biologically significant dose of zearalenone. However, as has been indicated, there is a considerable variation in sensitivity between species and the toxicity of zearalenone in man is unknown, but based upon data from other primates (Hobson *et al.* 1977) is probably low. Ueno and Kubota (1976) suggested that zearalenone was mutagenic to a recombination-deficient line of *Bacillus subtilis*, but this could not be confirmed by Wehner *et al.* (1978), using *Salmonella typhimurium*.

Schoental (1979) has suggested that zearalenone and other *Fusarium* mycotoxins may have a role in the aetiology of tumours of the digestive tract and gonads in animals and man, and there has been speculation (see Martin and Keen 1978) that dietary oestrogens might be implicated in the high incidence of cervical cancer in certain areas of Southern Africa, e.g. Swaziland and Lesotho.

Mouldy corn from areas of the Transkei associated with high and low incidences of oesophageal cancer has been examined for mycotoxins (Marasas *et al.* 1979). Pooled samples from these areas showed no significant differences in the extent and nature of the *Fusarium* species present. However, when four sub-samples of hand-selected, visibly *Fusarium*-infected kernels were analysed, significant differences in the nature and extent of the infection were observed. All of the sub-samples contained zearalenone (ranging from 1500 to 10 000 μg/kg) but the mean level of the samples from the high-incidence area was 5750 μg/kg compared to 2750 μg/kg from the low-incidence area. Even larger differences were noted in the levels of deoxynivalenol, being 250 μg/kg and 2500 μg/kg in the low-incidence and high-incidence areas respectively. The authors concluded that before the potential threat to human health of these *Fusarium* metabolites in mouldy corn could be evaluated more detailed information was needed on their chronic effects and on whether any additive or synergistic effects might occur.

Other compounds claimed to possess oestrogenic activity

Examination of table 1 reveals additional compounds which have been claimed, with varying degrees of supportive evidence, to be responsible for the oestrogenic activity of the individual plant species shown. For example, Stob (1983) has suggested that the hormonal activity of carrots (Ferrando *et al.* 1961) may be related to the presence of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (XXVIII, figure 10). However, this compound was isolated from cold-stored carrots and was apparently absent in the freshly harvested root (Sondheimer 1957). Little, if anything, is known about the effect of such storage on the uterotropic effect of this vegetable. Anethole (XXIX) was suggested by Zondek and Bergmann (1938) to be responsible for the oestrogenic activity of essential oils of fennel and anise, but more recent physiological studies on this compound (Sangster *et al.* 1984a, b) have not supported this. Three structurally related bitter acids, colupulon (XXX), lupulon (XXXI) and adlupulon (XXXII) have been identified in hops and proposed as the oestrogenic principles therein (Zenisek and Bednar 1960).

Feldman *et al.* (1982) described a protein in bakers' yeast (*Saccharomyces cerevisiae*) capable of binding 17β-oestradiol with high affinity; moreover, a chloroform extract of the same yeast cells was found to bind competitively to mammalian oestrogen receptor cells *in vitro*. Subsequently the same group (Feldman *et al.* 1984) showed this extract to possess uterotropic activity. If these findings are confirmed, high priority should be given to the isolation and identification of the active component(s), given the extensive use of this material in baking and fermentation. Only when its potency has

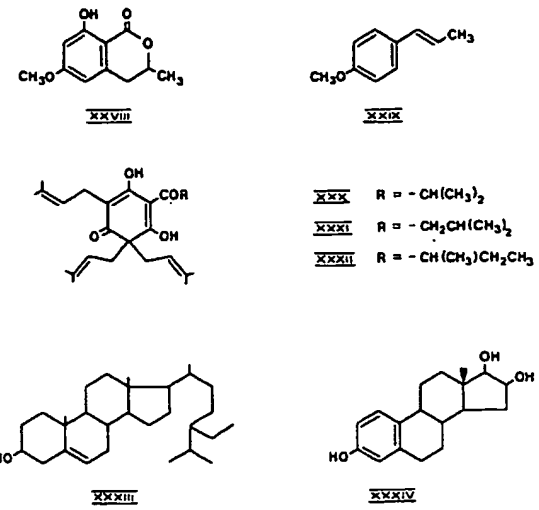


Figure 10.

been determined and its levels in food products have been established can the full significance of the above findings be ascertained.

According to Hassan *et al.* (1964), β-sitosterol (XXXIII) was one of the factors responsible for the hormonal activity of liquorice, although this has been questioned. Certainly if this and related compounds were to be confirmed as possessing such activity, then their ubiquity might well explain the hormonal properties of a range of common food plants, including onion and garlic and certain vegetable oils (Booth *et al.* 1960). Rather earlier, Costello and Lynn (1950) had tentatively identified the steroidal oestrogen, oestriol (XXXIV), as being present in liquorice. The role, and indeed the presence, of such steroidal hormones in the plant kingdom has been the subject of considerable controversy (Hewitt *et al.* 1980). As these workers have emphasized, early investigations into the hormonally active principles of plants were limited by relatively crude means of fractionation, isolation, characterization and bioassay. Consequently many of the initial claims for the occurrence of steroidal oestrogens in the plant kingdom were treated with scepticism. In 1966, Bennett *et al.* identified oestrone in date palm pollen by thin layer chromatography, and it was subsequently isolated from the same source (Amin *et al.* 1969). Pomegranate seed was claimed to be another source of this compound, with levels of 17 mg/kg being reported (Heftmann *et al.* 1966).

Whilst the presence of oestrone was confirmed by Dean *et al.* (1971), the measured levels were very much lower (4 μg/kg). 17β-Oestradiol could not be detected. In other cases (see Hewitt *et al.* 1980), workers were unable to isolate steroidal oestrogens from plant sources despite previous claims to the contrary. Such irreproducibility, limitations in analytical technique and methodology and the overriding concern that presence of such compounds, even when proved conclusively, might be a result of contamination meant that fundamental questions about the natural occurrence of such compounds in plants remained until recently (Van Rompuy and Zeevaar 1979), and the

failure of these workers to identify steroidal oestrogens in plant extracts using sophisticated modern techniques clearly identifies this area as a rewarding one for further interdisciplinary study. Work described in detail by Hewitt *et al.* (1980), using radioisotope incorporation studies and sensitive gas chromatography-mass spectrometric techniques, unequivocally revealed the presence of oestrone and oestradiol in French bean seedlings.

Residues of pesticides and insecticides may also be a source of uterotropically active compounds in the human diet. It has long been known that DDT and its analogues exhibit such activity (Fisher *et al.* 1952, Welch and Conney 1968) and recently Loeber and van Velsen (1984) have shown β-HCH, an isomer of lindane and a component of technical HCH, to have uterotropic activity. Although very weak (2×10^{-5} that of 17α-ethynyloestradiol), little is known about the effects of long-term exposure to trace amounts of such compounds.

Overview

Amongst the plants consumed by humans which have been reported to possess oestrogenic activity are onion, garlic, coffee, apple, parsley, sage, rhubarb, potato, radish, pea, cucumber, sugar beet, cabbage and mustard. These reports, originating from the early work of Dohrn *et al.* (1926) and Löve and Löve (1945), did not identify any of the active components, and were based upon methods of analysis which are now recognized to have limitations. Nevertheless it would seem desirable to re-examine some of these food plants using modern methods of analysis, in particular those like potato and cabbage, which are consumed regularly in relatively large amounts. The widespread use of vegetable oils also suggests that the claims that these are uterotropic be re-examined. Because of the evident variation in sensitivity to oestrogens exhibited by different species and strains of animal it would be desirable to standardize the uterotropic assay so that results from different laboratories and on different commodities/food plants could be more readily compared.

Inspection of table 6, taken from Verdeal and Ryan (1979), would seem to suggest that there is little risk associated with the intake of plant oestrogens. This is not necessarily the case, however, since little is known about the effects of long-term low-level exposure to these compounds (or their metabolites). Studies with human subjects would be desirable to determine whether or not normal levels of intake are associated with detectable physiological changes. This might provide objective predictions of the nature and extent of any changes which might occur in particular

Table 6. Human exposure to exogenous oestrogens.[a]

Source	Estimate of possible daily dose (μg diethylstilboestrol equivalents)
Morning-after pill	50 000
Birth control pill	2500
Post-hysterectomy replacement therapy	500–1000
Post-menopausal therapy	500
100 g beef liver (0·5 p.p.b. diethylstilboestrol)	0·05
100 g wheat (2 p.p.m. zearalenone)	0·2
20 g (d.w.) soyabean sprouts (70 p.p.m. coumestrol)	0·5
100 g French beans (2–10 p.p.b. oestradiol)	0·03–0·15

[a] Data from Verdeal and Ryan (1979) with permission.

'at-risk' sections of the population. Moreover, consideration should be taken of any medium or long-term changes in dietary habits which might be expected to increase the intake of such phytoestrogens; the increasing use of vegetable proteins in general and soya protein in particular and the introduction of soya milk products for infant feeding are two such examples (Setchell *et al.* 1984).

The importance of metabolic studies in determining the likely oestrogenic effect associated with the ingestion of isoflavones and resorcylic acid lactones is obvious, the effect depending as it does on the extent of that metabolism and the individual potencies of the metabolic products. The metabolism of the coumestans should be examined and the activities of the major isolated metabolites determined. Further studies should also be conducted on equol; for example, examining its effect *in vivo* and *in vitro*. Such studies will obviously depend upon the availability of methods of analysis for both the parent compounds and the metabolites. The examination of the possible carry-over of resorcylic acid lactones, and metabolites, following the feeding of *Fusarium*-infected diets or the implantation of zearalanol as growth stimulant would be desirable using such methods. The possible synergistic effect of different plant oestrogens or of plant and synthetic oestrogens should not be discounted (Kotsonis *et al.* 1975).

Attention should be given to programmes designed to limit or reduce the intake of plant oestrogens, whether by judicious selection of plant varieties or the optimization and improvement of agronomic, storage and processing conditions. Notwithstanding the inherent difficulties, a detailed study of the dietary factors associated with the high incidence of certain cancers in Southern Africa might provide useful information as to the role of dietary oestrogens. Indeed, a fuller understanding of the biological basis of the hormonal activity of the plant oestrogens considered above, particularly in farm animals and primates, would seem long overdue.

Future progress in this interesting and challenging area will largely depend upon the integrated efforts of workers from a variety of disciplines, including chemists, biochemists, toxicologists, pathologists, food technologists and plant breeders. As such it would seem to be particularly suitable for support from national and international food and health agencies.

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REPRODUCTIVE AND GENERAL METABOLIC EFFECTS OF PHYTOESTROGENS IN MAMMALS

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Key Words: Phytoestrogens, Mammalian reproduction, Reproductive hormones, Gonadal steroids, Luteinizing hormone, General Metabolism, Ovarian function, Reproductive neuroendocrinology.

INTRODUCTION

Historically, phytoestrogens were first investigated when it was noted that ewes that grazed Australian clover pastures for prolonged periods of time became sterile. It was found that the active agents in the clover that precipitated sterility were estrogenic (1). Later a similar phenomenon was observed to occur in the California quail during dry years, when phytoestrogen concentrations in available forage were increased (2).

Phytoestrogens are defined as plant substances that are structurally and functionally similar to the gonadal steroid 17β-estradiol (E_2) or that produce estrogenic effects (3). There are three main groups of nonsteroidal dietary estrogens. Phytoestrogens include the isoflavones (i.e., genistein, genistin, daidzein, biochanin A, formononetin, and pratensein) and the coumestans (i.e., coumestrol and 4'-o-methylcoumestrol). Mycoestrogens of the resorcylic acid lactone group (i.e., zearalenone and zearalenol) are also commonly found (4). The structural similarity between these substances, endogenous mammalian estrogens (E_2 and estrone), and potent synthetic estrogens (diethylstilbestrol) have been studied (Figure 1). Isoflavones, the monocarboxylic derivatives of the 15-C flavones, and coumestans contain central structures of 15 carbons. Both of these groups are derivatives of 3-phenylchroman (Figure 1) and thus may be considered a single family of compounds (5). The fungal resorcylic acid lactones and endogenous estrogens possess central structures of 17 carbons.

The similarity among these compounds has led investigators to study the possibility that phytoestrogens might act on physiological processes and behavioral patterns to alter reproductive performance (3). If reproductive effects occur, then these compounds might have a role in the evolutionary success of herbivores, perhaps making the difference between survival and extinction for some species. It is possible that phytoestrogens, through mimicry of endogenous animal estrogens, function as defensive substances by which plants diminish the fertility of herbivores which feed on the plants (6). In effect, the phytoestrogens may be seen as one of the many variables determining animal fitness for survival. This argument is supported by noting that animal species differ in their sensitivity to phytoestrogens (7). Some species are relatively resistant to the estrogenic effects of these compounds, while others may suffer sterility as a result of prolonged ingestion of phytoestrogens. We have hypothesized that phytoestrogen-induced physiologic and behavioral effects in mammals are significant factors in the reproductive and therefore evolutionary success of the consuming species. We have initiated our analysis of this broad hypothesis by reviewing the available data relevant to the reproductive and general metabolic effects of phytoestrogens in mammals.

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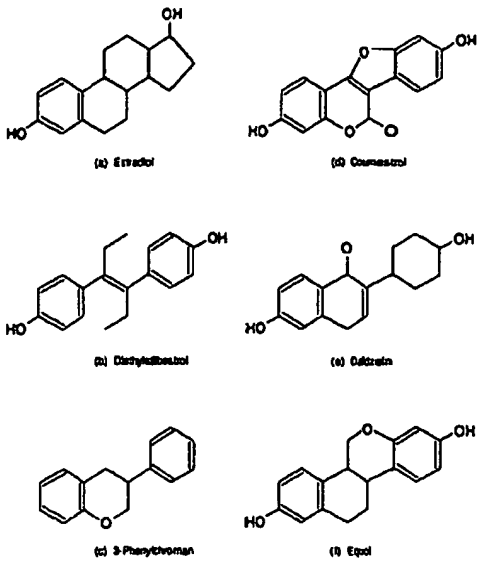

Fig. 1. Structure of common estrogens and phytoestrogens. The major physiological estrogen, estradiol (a), and the potent synthetic estrogen, diethylstilbestrol (b) are shown for reference. 3-Phenylchroman (c) is the phytoestrogen precursor compound to the coumestans such as coumestrol (d) and isoflavones such as daidzein (e). Equol (f) is an estrogenic metabolite produced within the gut from other phytoestrogens of the isoflavone group. (Modified from: Setchell, K. D. R. Naturally Occurring Non-steroidal Estrogens of Dietary Origin. In: Estrogens in the Environment I. A. McLachlan, ed. New York: Elsevier Press, 1985; 69–85.)

PHYTOESTROGEN EXPOSURE

Sources of phytoestrogens

Phytoestrogens are produced by numerous Leguminosae and grasses, including many plants commonly consumed by man and livestock (Table 1). The estrogenic components are found in differing amounts in all parts of the plant, including the seeds, the flowers, the leaves, the roots, and the fruits. Concentrations in each tissue depend on plant type (4,8).

Of particular interest regarding possible human exposure is the presence of phytoestrogens in marijuana and coffee. It had long been suspected that the estrogenic effects of marijuana were due to Δ^9-tetrahydrocannabinol (THC), the major psychoactive compound. Smoking of marijuana significantly suppresses luteinizing hormone (LH) levels during the human menstrual cycle and shortens both the menstrual cycle and the luteal phase (9). Since these results agree with observations in ovariectomized rhesus monkeys injected intramuscularly (i.m.) with THC, it was assumed that the menstrual cycle effects of smoke inhalation would be exclusively due to the THC content of the smoke (10). However, crude marijuana extract and condensed marijuana smoke compete with estradiol for estrogen receptors in the uterus of rats, while in vitro studies detected no binding of cannabinoids to estrogen receptors (11). These findings show that marijuana contains estrogenic substances that may be affecting reproductive processes via cannabinoid-independent mechanisms. Furthermore, apigenin, a derivative of flavonoid phytoestrogens found in crude marijuana, is a moderately potent inhibitor of estradiol binding to uterine estrogen receptors (11). Differentiation between the suppressive effect of THC on LH and the estrogenic effects of marijuana *per se* remains unclear.

Table 1. Some common plants that contain estrogenic substances

Alfalfa	Coffee	Oats	Rice
Anise	Date Palm	Orchard grass	Rye
Apple	Fennel	Palmetto grass	Sage
Barley	French Beans	Parsley	Sesame
Blue grass	Garlic	Peas	Soybean
Carrot	Green Beans	Pomegranate	Soya sprouts
Cherry	Hops	Potato	Wheat
Clovers	Liquorice	Rape	Yeast
	Marijuana	Red Beans	

Another plant product which is commonly ingested for pleasure rather than nutrition is coffee. Like marijuana, coffee contains weakly estrogenic constituents, evidenced by the estrogenic effects of increased uterine-to-body weight ratio and total uterine protein content following administration of coffee extracts by gavage (12). Ultraviolet absorbance spectroscopy suggests that whatever this active compound may be, it does not belong to one of the three major classes of dietary estrogens (e.g., flavonoids, coumestans, or resorcylic acid lactones). Thus, coffee may contain an estrogen precursor that requires metabolic activation or a structurally unrelated estrogenic compound.

Metabolism, distribution, and clearance

The relative potency of a phytoestrogen depends upon the target tissue, functional state of the target tissue, the animal species involved, and the route and pattern of delivery. In addition, the fami-

lies of estrogenic compounds that occur in plants can be modified by metabolism within the herbivore or even by gut flora prior to uptake. Dietary isoflavone phytoestrogens undergo bacterial modification in the gastrointestinal tracts of animals to yield equol, a weak, nonsteroidal phytoestrogen (8,13,14). Following ingestion of estrogenic plants, a temporary 50- to 1000-fold increase in urinary equol takes place, while insignificant traces of the initially consumed phytoestrogens appear in the urine. Noteworthy is that the major urinary product following the consumption of genistein and biochanin A is p-ethyl phenol, and formononetin consumption yields both daidzein and equol as the major urinary products (4). Furthermore, gut microflora (14) convert daidzein to equol which in turn is absorbed and enters the enterohepatic circulation. Notably, it appears that not all people have the ability to convert other isoflavones to equol. This may be due to the absence of bacteria capable of the conversion of precursors to equol (as is the case in the sterile gut of newborns), the composition (subpopulations) of intestinal microflora present, the intestinal transit time, pH, or redox potential. These factors may be influenced by diet, host immunity, medication use, etc.

Receptor activity and interaction with endogenous estrogens

Phytoestrogens exhibit binding to endogenous estrogen receptors. Binding of phytoestrogens to estrogen receptors is supported by the finding that the larger the dose of phytoestrogen given an organism, the greater the displacement of bound tritiated (^{3}H) E_2 (15). It has also been reported that at very high dosages, all phytoestrogens exhibit more than 80% competitive binding to renal tumor cytosolic estrogen receptors (16). The structural requisites for estrogen receptor binding are met by phytoestrogens. For example, equol possesses a potency on the order of 10^{-3} the estrogenic activity of E_2 and contains a phenyl substituent also present in E_2 and in DES (Figure 1). The substituent considered to be a requirement for estrogenic activity is a hydroxyl group in the same position as the hydroxyl group in the benzene ring of E_2 (14). Another structural similarity which facilitates estrogen receptor binding activity of equol and other phytoestrogens is that the distance between C-3 and C-17 in E_2 is about equal to that between the two hydroxyls in equol.

Considering the large quantities of phytoestrogens ingested by many mammals including man, functionally significant estrogen receptor occupancy by phytoestrogens occurs. Since no phytoestrogen has receptor affinity equal to that of E_2 and the degree of DNA stimulation due to phytoestrogens appears to be substantially less than that evoked by E_2 (8), phytoestrogen actions could be either estrogenic or anti-estrogenic. In a relatively hypoestrogenic individual, receptor occupancy by weak (exogenous) estrogens would likely produce estrogenic effects, while in a normally estrogenized individual, large amounts of weak estrogens might diminish the effective estrogenic activity by competition with E_2.

REPRODUCTIVE EFFECTS IN MAMMALS

Phytoestrogens have been shown to influence virtually every aspect of the mammalian reproductive process via effects on the morphology and physiology of reproductive organs and alteration of sexual behavior. The changes may be reversible or irreversible, depending on the duration and dose of exposure to the phytoestrogens.

Cervix

A pubertal pattern of cell differentiation has been noted in ewes rendered sterile by chronic ingestion of phytoestrogens (17). Among these changes, the cervix assumes a uterine pattern. Folds present in the cervix fuse, resulting in loss of cervical crypts, and the cells of the lamina propria become like those of the uterine stroma. Furthermore, glands having histochemical reactions reminiscent of uterine glands become plentiful in the cervix. Such an increase in abnormal glands may be responsible for the different composition which the cervical mucus takes in sheep with "clover disease." At low phytoestrogen dosage, the cervical mucus has a lower viscosity, not due to a higher water content, but rather due to a decreased concentration of glycoprotein — the component of mucus that affords its consistency. The level of glycoprotein seems to respond to the duration of exposure to the phytoestrogen rather to the dosage of the agent. This change in the cervical mucus compounds the anatomical compromise of the cervix such that the cervical reservoir for sperm in the ewes is greatly reduced. Since sperm recovered from the cervices of clover-affected ewes exhibit decreased motility (17), it appears that the phytoestrogen effect makes the mucus relatively "hostile" in the classic sense of cervical factor infertility. Such spermatoxicity is not understood in general nor in this specific case.

At higher phytoestrogen dosage, both higher volume and water content of cervical mucus are

observed in ewes (17,18), thus indicating that both cervical glycoprotein production and water excretion in the mucus are affected.

The cervical effects of phytoestrogens likely depend upon estrogen receptor mediation. In ewes, phytoestrogen treatment increases the rate of protein and glycoprotein synthesis and the number of estrogen binding sites in the cervix, but binding affinity remains unchanged (19). This finding implies that exogenous estrogen not only occupies the available binding sites, but stimulates the local production of more sites. Such receptor "up-regulation" may make the tissue more sensitive to estrogen action, and, if estrogen exposure continues, the cervical alterations would become more exaggerated.

Uterus

Pronounced uterine effects of phytoestrogens are also observed. The most notable uterine change that occurs is a marked increase in its weight relative to body weight, which constitutes the classic bioassay for estrogen action. A dose-dependent uterine weight increase is precipitated by acute administration of an extract of the Indian herb *Achyranthes aspera* in rats and hamsters at contraceptive dosage (75 mg/kg) and with as little as 1/20 this dosage (20). Similar results have been observed in mice, rats, and hamsters with only 1/40 contraceptive dose of ferujol extract (21). Stob (4) suggests that this hypertrophy of the uterus is the result of "typical estrogenic mechanisms," implying estrogen-receptor mediation. However, a more complex response to daily s.c. injection of female lambs with the phytoestrogen β-sitosterol has been reported, in which uterine weight increases for the first two weeks of treatment but markedly decreases over the next six-week period (22). Plausible explanations for such biphasic results include receptor "down regulation" and induction of metabolic enzymes with enhanced clearance of β-sitosterol. Similar results were obtained using ovariectomized ewes as the model (23).

Another manifestation of the uterotropic effect of phytoestrogens is seen in ewes suffering from infertility due to prolonged exposure to these agents. A marked increase in activity of some uterine enzymes and uterine DNA, protein, and glycoprotein synthesis occurs in such sheep (19). This observation indicates that at least a portion of the uterine weight gain is true hypertrophy rather than simply edema. At the same time, lower levels of lipids within the uteri of sheep fed phytoestrogen suggest inhibition of synthesis or increased utilization of lipids within this organ (22). Thus phytoestrogens may be affecting different enzymes in different fashions, stimulating the activity of some while blocking the action of others. It is noteworthy that the uterine RNA-to-DNA ratio decrease that occurs following ovariectomy is smaller in clover-affected than in normal ewes. This response is accompanied by less regression of the uterus in clover affected ewes than in controls. These findings indicate that phytoestrogenic action may be mediated via differentiations similar to those induced by hormonal steroids during fetal development (24).

Gross structural lesions of the uterus may also result from phytoestrogen exposure and could account for some instances of permanent sterility. Lesser lesions entail the proliferation of cystic endometrium, myometrial fibrosis, and endometrial fibrosis (13). These lesions could certainly compromise normal implantation of the conceptus. The most severe structural failure, complete uterine prolapse, is known to occur in some species following ingestion of some dietary estrogens (mycoestrogens) and obviously disrupts the reproductive process.

It is not clear whether phytoestrogens play any role in pregnancy wastage, but some plant preparations have been used as abortifacients. *Achyranthes aspera*, a common Indian herb claimed to possess abortifacient activity, did induce abortion in mice and rabbits, but failed to show similar effects in rats (20). It is uncertain whether a phytoestrogen is the active agent of *Achyranthes* that brings about abortion, but support for that possibility derives from the finding that miroestrol, a phytoestrogen from a legume tree root, is used by Burmese and Thai women in plant extract form to induce abortion (25). The mechanism for such an abortifacient action of these compounds is unstudied and any effects of phytoestrogens on uterine contractility *per se* have not been determined in either the gravid or nongravid state.

Phytoestrogen effects on uterine function may relate to alterations in activity of several enzymes. Under normal circumstances, oxidative enzymes in the uterus show slight reactions in the endometrium and uterine glands, but after administration of β-sitosterol, these weak reactions are curtailed (22). Such an inhibition of oxidative enzymatic activity in the uterine endometrium and glands may reduce local energy production due to an inability to replenish NAD+ and NADP+. This circumstance would diminish the ability of the uterus to contract and might decrease secretory capabilities of the uterine glands.

Alkaline phosphatase in the uterine tissue of ewes also responds to β-sitosterol in a biphasic pat-

tern. Alkaline phosphatase activity increases over the first two weeks of daily β-sitosterol injections and decreases over the second two weeks of injections (22). This disturbance in alkaline phosphatase activity may alter cell permeability and transport of nutrients by uterine cells.

Acid phosphatase activity in the uterus decreases with increasing dose and time of daily β-sitosterol treatments over an eight-week span (22). Such an inhibition would decrease free phosphorous, and may relate to the more general observation of decreased plasma phosphorus levels in exposed animals.

Uterine cholinesterase activity also decreases following β-sitosterol treatment, as evidenced by its diminished activity towards acetylthiocholine (22). This inhibition of activity is accompanied by a downward shift in sodium ion transport and decreased sodium in the uterine luminal fluid. It is not clear whether effects on sodium transport and cholinesterase activity are coincidental or truly associated processes in this instance.

Ovaries

While many anatomical effects of phytoestrogens have been described, physiologic changes in the reproductive tract are more subtle, but perhaps more consequential. Ovarian cyclicity may be disrupted by phytoestrogen exposure in mammals and birds (2,14,25,26), but interruption of ovulation due to short-term phytoestrogen ingestion is reversible (26). It is plausible that human vegetarians may have ovulatory dysfunction but suffer no other obvious physiologic abnormalities due to their diets (14). Abnormalities of ovulation may be due to direct ovarian actions since administration of β-sitosterol to ewes inhibited follicular development and altered the size distribution of follicles (22). Follicles were observed to show degeneration with intrafollicular hemorrhage and the development of shrivelled oocytes with lipid inclusions. The suggestion of a direct ovarian action of phytoestrogens in perturbing follicular maturation may be supported to some extent by a study which showed that in rats intraperitoneal administration of an extract from a plant species known to contain high concentrations of phytoestrogens inhibited follicular maturation (26). Obviously, these studies cannot distinguish between direct ovarian and indirect effects on follicular growth.

More direct evidence that the follicle may be a site of phytoestrogen activity derives from in vitro cultures of bovine granulosa cells. In this system, lower dosages of genistein and biochanin A increased progesterone synthesis while higher dosages inhibited progesterone synthesis (27). Since progesterone is essential in the establishment and maintenance of pregnancy, such an inhibition of progesterone production would be a plausible explanation for both failure of conception and early pregnancy wastage.

The possibility that phytoestrogens might be toxic to oocytes or early embryos was suggested in a single study (7). Mice fed coumestrol and then mated produced degenerate embryos exhibiting unevenly distributed cytoplasm and lack of symmetry in size among blastomeres, suggesting alterations in cleavage rates. Extensive vacuolization found in the ova also suggests that failure of fertilization of these ova may account for part of the observed decrease in litter size in mice fed coumestrol.

The activities of two ovarian enzymes appear to be influenced by phytoestrogens. First, low doses of phytoestrogen inhibit 17,20-lyase in bovine granulosa cells (27). This effect could profoundly alter the pattern and capacity of the steroidogenic pathways within the follicle or corpus luteum. The precise mechanism by which this effect occurs is unproven. Second, alkaline phosphatase in the ovaries is affected by phytoestrogen exposure (22). While the overall alkaline phosphatase activity is about equal in the ovaries of β-sitosterol-treated and control ewes, the control ewes show an intense reaction in the zona pellucida with a weak reaction in the interstitial tissue. Treated ewes exhibit an opposite response. Thus, a reversal of activities is seen where phytoestrogen is acting both to stimulate and to inhibit the same enzyme in two different sites within the ovary. While a mechanism for this action is not known, such changes in the activities of ovarian enzymes might compromise ovulation and increase the incidence of follicular degeneration in animals treated with phytoestrogens.

CNS/pituitary

Some phytoestrogen effects on ovarian function appear to result from indirect action on the secretion of gonadotropic hormones (7). In this context, there are four possible mechanisms of phytoestrogen action: 1) they are E_2 agonists, 2) they are E_2 antagonists, 3) they act as both E_2 agonists and antagonists, and 4) they act in a nonestrogenic capacity. Available information best supports the third of these possibilities (mixed agonist-antagonist effects). The site of phytoestrogen action could be the CNS (especially hypothalamus), the pituitary, or the gonad (see previous section).

The effect of intraperitoneal injection of phytoestrogen-rich *Dieffenbachia amoena* extract in rats on LH, follicle-stimulating hormone (FSH), prolactin (PRL), progesterone, and E_2 have been studied (26). In treated rats, levels of LH, FSH, and progesterone increased for doses of 2.5, 5.0, and 10.0 mg/kg of extract, while the levels of PRL and E_2 decreased at the same dosages. Progesterone levels showed a biphasic response, increasing at low doses of the extract (26), but decreasing at higher doses (27). Since no obvious single mechanism would explain all of these pituitary and ovarian hormonal changes, the extract may contain more than one endocrinologically active substance, or more than one site or mechanism of action might be involved.

There are data to suggest that phytoestrogens act both at CNS and pituitary levels to alter gonadotropin secretion. In both ovariectomized ewes (23) and intact clover-affected ewes (17), the best explanation for the impairment of gonadotropin secretion was a hypothalamic/CNS action. In particular, in clover-affected ewes, an LH surge could not be elicited by exogenous E_2 administration (consistent with loss of positive feedback), but the LH secretory response to exogenous gonadotropin-releasing hormone was normal (17), suggesting no pituitary effect. Our own data (28) show that acute phytoestrogen administration can alter GnRH-induced LH secretion in ovariectomized rats and thus suggest that the pituitary may be a site of phytoestrogen action in other situations.

Interactions between reproductive effects of phytoestrogen exposure and photoperiod in seasonal breeders have been investigated. In normal intact ewes, the frequency of LH pulses and plasma LH concentration are higher during breeding season than during anestrus season. In clover-diseased ewes, the frequency of LH pulses and LH concentration during breeding season are nearly the same as in normal ewes. In contrast during anestrus season, these LH pulse parameters remain at the high level of breeding season in clover-affected ewes, rather than decreasing as in normal ewes (18). These results suggest that a dissociation of normal photoperiod controls from the LH pulse generator may result from prolonged phytoestrogen exposure.

In ovariectomized ewes given estradiol implants, LH pulse frequency and amplitude vary seasonally, rather like the pattern seen in intact ewes. This seasonal variation in LH pulse frequency in ovariectomized ewes could depend upon extraovarian steroids from the adrenal glands, other intrinsic photoperiod-dependent CNS functional changes, or dietary estrogens. Results from one study suggest that dietary coumestrol decreases the amplitude of LH pulses but fails to affect the frequency of LH pulses or FSH concentrations during the breeding season (23). During anestrus, coumestrol does not alter any of these variables. Thus, coumestrol could only be partially responsible for the seasonal decrease in LH pulse frequency in ewes.

Sexual behavior

Changes in sexual behavior due to phytoestrogen exposure parallel the known physiologic effects. Clover-diseased ewes are slower than normal ewes to exhibit estrus behavior in response to either a single or several daily doses of E_2 (17,29,30). Accompanying the delayed estrus is a retarding of the first mount of the ewes by the ram, although the number of days on which the ewes allowed the ram to mount them does not significantly differ from controls. A delay of estrus in mice fed coumestrol also occurs (7), implying an antiestrogenic effect.

Apparent defeminization of the sexual behavior response following consumption of phytoestrogens is displayed by clover-affected ewes. These ewes show aggressive behavior, such as challenging and head bunting of rams and other ewes, sooner than control ewes following administration of testosterone (17). At the same time the ewes are slower in showing female behavior, such as standing to be mounted by a ram. Furthermore, clover-affected ewes exhibited less soliciting behavior than normals. However, the number of ewes that stood to be mounted decreased equally over the five-week period during which daily testosterone injections were given (30). Relative to controls, clover-diseased ewes exhibit a significantly greater degree of courting behavior 28 but not 21 days following treatment with testosterone. Other courting behaviors that are less hormonally dependent, such as anal and genital sniffing by the ewes, are not altered (17,30). While mechanisms for these behavioral effects are not known, we do know that females and males have similar numbers of estrogen binding sites in the hypothalamus, but estrogen-receptor complexes appear to have shorter nuclear acceptor occupancy in males than in females (31). Behavioral changes in clover-affected ewes could result from a change as simple as a decrease in nuclear acceptor occupancy by estrogen-receptor complexes.

E_2 causes a dose-dependent increase in the incidence and duration of hormone-dependent behaviors in ewes (Table 2), whereas E_2 has no effect on hormone-independent behaviors (30). The E_2-

Table 2. Estradiol-dependent and -independent behaviors in ewes

Hormone-dependent behaviors	Hormone-independent behaviors
Active soliciting	Squatting
Standing for mounting	Looking over shoulder
Allowing ram to mount	Tail fanning
	Kicking

induced behaviors occur less in phytoestrogen-affected ewes than in normals, while E_2 independent behaviors occur with equal frequency in controls and clover-diseased ewes. Since general behavior appears normal but female sex-specific behavior is compromised in phytoestrogen-treated ewes, reproductive success could be compromised on a behavioral basis. The relationship of phytoestrogen-induced anatomic changes in the external genitalia and sexual behavior is not defined, but coital biomechanics could be altered as a result of such end-organ effects. While vulvar and vaginal hypertrophy has been noted in various animals, masculinization has been observed in ewes (17) with clitoromegaly and fusion of the ventral commissure. Upon removal from estrogenic pasture, these changes do not reverse and could, therefore, permanently alter sexual function.

Phytoestrogenic effects in males appear to be consistent with expectations for exogenous administration of bioactive estrogen. Coumestrol increases teat length in wethers (23) and stimulates mammary hypertrophy in intact males. Rams grazed on estrogenic clover have reduced sperm counts (14), but it is not clear whether fertility is affected.

GENERAL METABOLIC EFFECTS IN MAMMALS

Protein synthesis

Some data suggest that phytoestrogens affect levels of plasma proteins. The effects of β-sitosterol on plasma concentrations of albumin, alpha-globulin, beta-globulin, gamma-globulin, and fibrinogen have been studied (32). Normal functions of these proteins are indicated in Table 3 (33). Even though total plasma protein concentration in mice is unaffected by s.c. administration of β-sitosterol, daily 25 to 100 μg injections of the agent increase four of the plasma proteins, but significantly decrease the gamma-globulin complex. The mechanisms of action of phytoestrogens in this system

Table 3. Plasma protein fractions affected by β-sitosterol*

Protein	Function	Effect of β-Sitosterol
Serum albumin	Regulation of blood volume; transport of fatty acids	Increase
Alpha-globulins	Transport of lipids, thyroxine, adrenal cortical hormones, and copper	Increase
Beta-globulins	Transport of lipids, iron, and hemes	Increase
Gamma-globulins	Act as most of the circulating antibodies	Decrease
Fibrinogen	Precursor to fibrin of blood clots	Increase

*(See reference 32).

are not established. It is likely that the phytoestrogens stimulate hepatic protein synthesis but inhibit production of gamma-globulins by lymphoid tissues. It is possible that the increased alpha-globulin concentration is a compensatory occurrence to erythrocyte count reduction that occurs following administration of β-sitosterol, thereby maintaining normal blood viscosity in the absence of normal erythrocyte concentration. The increase in the beta-globulin–fibrinogen complex appears to be correlated with its affinity for binding phosphorus. This affinity increases in response to β-sitosterol (32).

Enzyme activity of the liver

Phytoestrogens influence enzymes in nonreproductive as well as reproductive tissues. A relation between diet and synthesis of three enzymes in the liver of cheetahs has been shown. The affected enzymes, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase, decrease in amount when cheetahs are taken off a diet high in soya bean content (thus high in phytoestrogen content) and given a chicken diet (13).

Inorganic plasma constituents

Phytoestrogens induce mineral changes in the blood. Subcutaneous injections of 25, 50, 75, or 100 μg of β-sitosterol increase calcium levels in mice, while doses of 5 or 10 μg of the phytoestrogen have no effect on calcium levels (34). Since E_2 inhibits bone mobilization, β-sitosterol may act by causing a decrease in E_2 levels via inhibition of gonadotropin secretion from the pituitary. Decreased ovarian E_2

production might then result in increased bone mobilization and increased serum calcium. Surprisingly, blood plasma phosphorus levels decrease following administration of 5 to 75 μg doses of β-sitosterol in mice, but show little change in response to a 100 μg dose (34). Decreases in phosphorus could be due to an enhanced rate of storage in an extravascular compartment, increased utilization of phosphorus by tissues, or increased renal clearance.

While β-sitosterol doses of less than 5 μg fail to change plasma magnesium levels, higher doses decrease plasma magnesium and increase both hepatic and intramuscular magnesium (34). Since magnesium is a smooth muscle relaxant, changes in uterine or tubal smooth muscle motility could result indirectly from this phytoestrogen action.

PHYTOESTROGENS IN HUMAN DISEASE

Deleterious roles

Phytoestrogens have been suggested to play both deleterious and beneficial roles with regard to illness. In the diets of cheetahs, phytoestrogens cause vascular hepatic lesions, in which the centrilobular and sublobular hepatic veins are partially or totally occluded (13). The possibility of human hepatic dysfunction must therefore at least be considered.

Vascular disease may be correlated with the consumption of dietary phytoestrogens (35). Coronary heart disease has been suggested to be associated with phytoestrogens consumed indirectly through the milk of cows; that is, the lactating cow consumes the phytoestrogens while grazing and, in turn, phytoestrogens in cow's milk are consumed by humans. One basis for this proposal is that phytoestrogens have more structural similarity to DES, a potent synthetic estrogen found to have atherogenic properties, than to endogenous estrogens such as E_2. The higher rate of coronary heart disease in human males might be explicable in part if human females are found to be better able to metabolize and excrete phytoestrogens.

Dietary estrogens could be a factor in cancer initiation in hormone responsive tissues, but no such instances have been demonstrated. Certainly phytoestrogens bind to both rat and human mammary tumor tissue and show competitive binding for mammary tissue E_2 receptors (15) raising the possibility of stimulation of estrogen-dependent neoplasms.

Beneficial roles

Estrogens have two opposing effects on cancer, depending on dosage. Large doses inhibit breast cancer tumor development and suppress growth of tumors already present, but small doses seem to promote tumor development and stimulate growth (36). This duality extends to phytoestrogens. Phytoestrogens may stimulate or inhibit tumor growth (8,14). One mechanism by which phytoestrogens may manifest their antitumor effects is blockade of estrogen receptors and uncoupling of receptor-mediated response. Thus the ability of endogenous estrogens to support tumor growth would be reduced. Indirect demographic support for a phytoestrogen-mediated reduction in cancers of hormone-responsive tissues might derive from the observation that women in countries consuming vegetarian diets have a lower incidence of breast cancer than in societies where a meat and vegetable diet is consumed (37).

Phytoestrogens may have antiviral and fungicidal properties (37), but a mechanism is not known. Support for the notion that this group of compounds could have such properties may lie in noting that the antifungal drug, ketaconazole, is also a potent inhibitor of some steroidal enzymes.

Plant estrogens have been implicated in the reduction of serum cholesterol levels in humans and animals with hypercholesterolemia. Such action is likely related to the role estrogens play in the metabolism and interaction of lipoproteins with regulation of cholesterol (8).

A final beneficial phytoestrogenic effect is alleviation of vasomotor symptoms in menopausal women. Historically the Chinese have used herbal medicine to treat "hot flushes." These herbal medications work as well as Premarin (an equine conjugated estrogen) in the mitigation of these symptoms in women with natural menopause (38). Similarly, the mycoestrogen, zearalanol, has been reported to reduce the incidence of hot flushes in women with surgical menopause (4). These effects would be consistent with the expected estrogenic properties of these compounds.

CONCLUSION

Phytoestrogens influence mammalian reproductive processes and can thereby compromise the reproductive success of individual mammals and possibly function as a selective environmental factor for populations. While phytoestrogens have a few propitious effects, the majority of the effects are nocuous. These compounds act through their similarity to endogenous estrogens and compete with the endogenous estrogens for binding sites.

Short-term effects of phytoestrogens seem to result from their mixed agonist–antagonist effects on estrogen-mediated processes in mammals. Since long-term exposures can produce persistent, even permanent anatomic, physiologic, or behavioral changes, phytoestrogens must affect the differentiation of some reproductive tissues and irreversibly alter the integration of mammalian reproductive processes in susceptable species.

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chen kommt es jedoch zu einer vollständigen Down-Regulation der entsprechenden Rezeptoren in der Hypophyse und damit zu einer chemischen Kastration (Abnahme der Testosteronproduktion und Spermiogenese).

Indikationen. Die synthetischen Gonadoliberine sind zur palliativen Behandlung des fortgeschrittenen Prostatakarzinoms indiziert. Goserelin kann darüber hinaus auch beim Mammakarzinom gegeben werden.

Dosierung. Von *Buserelin* gibt man 7 Tage lang 3mal täglich (in achtstündigen Abständen) 0,5 mg s.c. Danach wird auf nasale Applikation umgestellt (3mal täglich vor und nach den Mahlzeiten je 0,1 mg in jedes Nasenloch).

Goserelin steht als Implantat zu 3,6 mg zur Verfügung, das Dosierungsintervall beträgt 28 Tage. Von *Leuprorelin* und *Triptorelin* werden täglich 0,2 mg bzw. 0,5–1 mg s.c. injiziert.

Nebenwirkungen. Vorteilhaft im Vergleich zur Behandlung mit Oestrogenen (s.u.) ist die geringere (bis fehlende) Feminisierung und das erniedrigte Risiko kardiovaskulärer Komplikationen.

Zu Beginn der Therapie des Prostatakarzinoms muß bei Patienten, die vorher nicht mit Hormonen behandelt wurden; infolge des vorübergehenden Anstiegs des Testosteronblutspiegels mit einer Verstärkung der Tumorsymptome, meist mit vermehrten Schmerzen im Bereich der Knochenmetastasen, gerechnet werden. Diesen kann durch die vorübergehende Gabe von Antiandrogenen (s. S. 680) vorgebeugt werden.

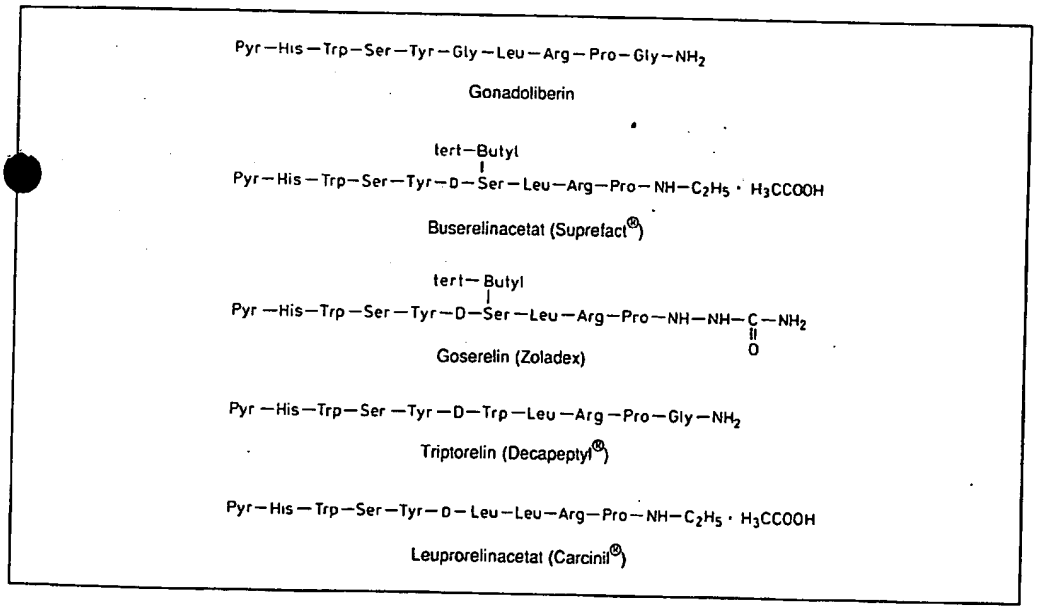
Als weitere unerwünschte Wirkungen im Verlauf der Behandlung treten bei sehr vielen Patienten Störungen der Libido und/oder Impotenz sowie in etwa 40% der Fälle Hitzewallungen auf. Ferner kann es zu Haut- und Schleimhautveränderungen kommen.

10.5.2 Oestrogene und Antioestrogene

10.5.2.1 Oestrogene

Oestrogene wurden beim Prostatakarzinom früher viel verwendet, da durch Oestrogengaben das Tumorwachstum verzögert und die Metastasenbildung gehemmt werden. Gleichzeitig werden die Schmerzen gelindert. Wegen gehäufter kardiovaskulärer Komplikationen unter der Oestrogentherapie sowie infolge der Einführung von Gonadoliberin-Analogen und Antiandrogenen wird die Indikation zur Gabe von Oestrogenen bei der Behandlung des Prostatakarzinoms heute zurückhaltender gestellt. Eine einmal begonnene Oestrogenbehandlung muß ohne Unterbrechung als Dauertherapie durchgeführt werden.

Daneben haben sich Oestrogene auch in einigen Fällen beim Mammakarzinom nach der Menopause



Die *Schleimhaut der Cervix uteri* nimmt nur wenig an den beschriebenen zyklischen Veränderungen teil. Bedeutsam ist aber, daß sich im Verlaufe des Zyklus die *Konsistenz des Zervixschleimes ändert.* Kurz vor der Ovulation tritt als Zeichen einer *Oestrogenwirkung* eine *Verflüssigung* des Schleimes ein, die zur Zeit der Ovulation ihren Höhepunkt erreicht. Der Schleim wird „spinnbar", d.h. er läßt sich zu langen Fäden ausziehen und ist für Spermien leicht durchgängig. Unter dem *Einfluß von Progesteron* wird der *Schleim* in der 2. Zyklusphase wieder *zähflüssiger* und behindert die Spermienpassage.

Dysmenorrhoe. Eine Dysmenorrhoe liegt vor, wenn Frauen während der monatlichen Regel ernsthaft beeinträchtigt werden und ihren beruflichen oder häuslichen Aufgaben nicht voll nachkommen können. Man unterscheidet eine *primäre* von einer *sekundären* Dysmenorrhoe. Bei der primären Dysmenorrhoe sind die Menstruationsblutungen *von Anfang an schmerzhaft,* bei der sekundären Dysmenorrhoe tritt die Schmerzhaftigkeit *erst in späteren Jahren* auf. *Lokale* dysmenorrhoische Beschwerden äußern sich in ziehenden Schmerzen im Rücken oder Unterleib, die sich bis zu heftigen Koliken steigern können. Daneben klagen die Frauen über allgemeines Unwohlsein, Appetitlosigkeit, Kopfschmerzen, Reizbarkeit, Herzklopfen u.a.

Als *Ursachen* für eine Dysmenorrhoe kommen Endometriose, Entzündungen oder Tumoren der Gebärmutter (mechanische Dysmenorrhoe), prämenstruelle Auflockerung des Beckenrings (statisch bedingte Dysmenorrhoe) oder psychische Faktoren, z.B. unerfüllter Kinderwunsch, berufliche Enttäuschungen usw. (psychisch bedingte Dysmenorrhoe), in Betracht.

Amenorrhoe. Unter einer Amenorrhoe versteht man das *Ausbleiben der Regelblutung.* Eine *primäre Amenorrhoe* liegt vor, wenn nach vollendetem 18. Lebensjahr noch keine Menarche (erste Regelblutung) eingetreten ist. Von einer *sekundären Amenorrhoe* wird gesprochen, wenn Menstruationen zunächst erfolgten, dann aber längere Zeit ausbleiben. Ferner muß zwischen einer *physiologischen* und einer *dysfunktionellen Amenorrhoe* unterschieden werden.

Eine physiologische Amenorrhoe tritt während der Schwangerschaft, im Wochenbett und in der Stillzeit auf.

Häufigste Ursache einer dysfunktionellen Amenorrhoe ist eine Ovarialinsuffizienz. Die Störung kann dabei sowohl im Hypothalamus-Hypophysen-System als auch in den Ovarien selbst liegen.

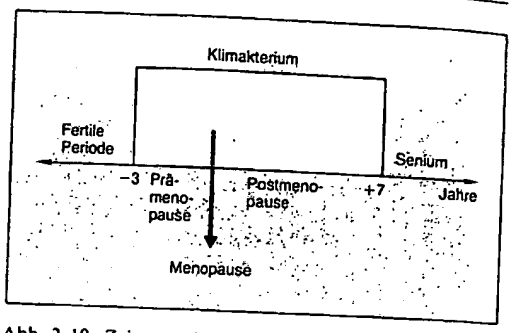


Abb. 2-19. Zeitraum des Klimakteriums

Klimakterium. Während des Klimakteriums, den sog. *Wechseljahren,* d.h. in der Übergangsphase von der vollen Geschlechtsreife in das Senium (s. Abb. B 2-19), stellen die weiblichen Keimdrüsen ihre Funktion ein. Diese erlischt im Mittel mit 49±3 Jahren. Zunächst werden die Menstruationsblutungen unregelmäßiger und schwächer; Ovulationen und Gelbkörperbildungen bleiben aus. Entsprechend dem massiven Abfall der Oestrogen- und Gestagenspiegel steigt für einige Jahre die Gonadotropinausschüttung stark an.

Als **Menopause** wird der Zeitpunkt der letzten Menstruation, als *Prämenopause* der Zeitraum drei Jahre vor und als *Postmenopause* der Zeitraum 7 Jahre nach der Menopause bezeichnet.

Bei etwa zwei Drittel der Frauen ist das Klimakterium mit Beschwerden, den sog. klimakterischen Ausfallserscheinungen, verbunden. Diese beruhen vor allem auf dem *Abfall der Oestrogensekretion.* Sie äußern sich in Form von

- *vegetativen Symptomen* (Hitzewallungen, Schwindel, Tachykardien, Schwitzen u.a.),
- *psychischen Symptomen* (Angstgefühlen, depressiver Verstimmung, erhöhter Reizbarkeit, Konzentrationsschwäche u.a.) und
- *metabolischen Dysfunktionen* (Osteoporose, Hyperlipoproteinämien, Hautatrophie u.a.).

Durch zyklusgerechte Substitution mit Oestrogenen und Gestagenen ist eine wirksame Therapie dieser Beschwerden möglich. Besondere Bedeutung kommt dabei der Prophylaxe der Osteoporose zu.

2.8.1.3 Hormonale Steuerung von Schwangerschaft, Geburt und Laktation

Hat die Befruchtung eines Eies stattgefunden, gräbt es sich mit Hilfe proteolytischer Enzyme in das Endometrium ein und wächst zum Embryo heran. Die

Ernährung des Embryos erfolgt über die *Plazenta,* die gleichzeitig eine hormonale Steuerfunktion übernimmt. Für diese Aufgabe sind zwei Hormone zuständig: das *Choriongonadotrophin* (*HCG* = *H*uman-Choriongonadotrophin), das vorwiegend in den ersten Schwangerschaftsmonaten gebildet wird, und das *Chorionmammotropin* (*CS* = *C*horion-*S*omatomammotropin), das u.a. die Brustdrüsen beeinflußt (s.u.).

Unter dem Einfluß der Plazentahormone kommt es zu einer *Vergrößerung des Corpus luteum,* das im ersten Monat einen großen Teil des Ovars einnimmt. *Die Oestrogen- und Progesteronspiegel bleiben hoch.* Daher wird das Endometrium nicht abgestoßen: Die *Menstruation bleibt aus.* Gegen Ende des ersten Schwangerschaftsmonats bildet sich das Corpus luteum zurück. Zu diesem Zeitpunkt hat die *Plazenta* selbst die Oestrogen- und Progesteronbildung aufgenommen, die für die Erhaltung der Schwangerschaft erforderlich sind.

Für die *Auslösung des Geburtsvorganges* werden verschiedene hormonale Faktoren verantwortlich gemacht, u.a. Abfall des Progesteronspiegels, Steigerung der Oxytocinabgabe, Anstieg der Plasma-Prostaglandine. Welchem Faktor die entscheidende Bedeutung zukommt, ist z.Zt. noch ungeklärt.

Die *Laktation* (Milchproduktion und -sekretion der Brustdrüse) wird ebenfalls hormonal gesteuert. Während der Schwangerschaft bilden sich unter dem *Einfluß der erhöhten Oestrogen- und Gestagenspiegel unter Mitwirkung von Chorionmammotropin* die *distalen Alveolen und Lobuli der Brustdrüse* aus. Die nach der Geburt einsetzende *Milchproduktion* wird durch *Prolactin* stimuliert. Die *Milchfreisetzung* schließlich erfolgt, ausgelöst durch den Saugreiz, unter Vermittlung von Oxytocin (s. S. 331 f.).

2.8.1.4 Oestrogene (Estrogene, Follikelhormone)

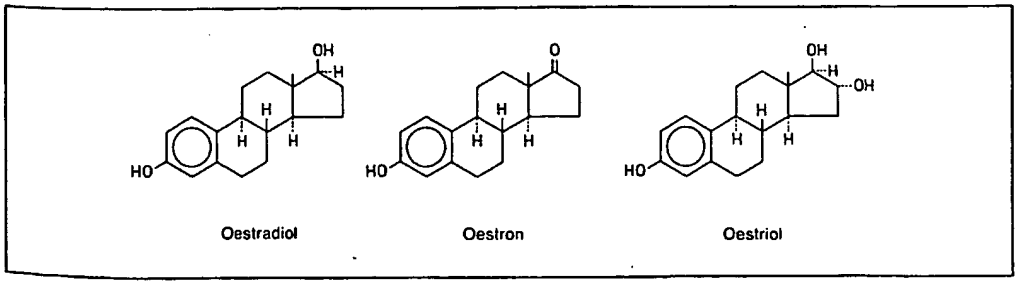
Die Oestrogene unterscheiden sich von den anderen Steroidhormonen grundsätzlich durch ihren aromatischen Ring A. An der phenolischen Hydroxylgruppe in 3-Stellung ist eine Salzbildung und damit die chemische Abtrennung der Oestrogene von den übrigen Sexualhormonen möglich. Auf diese Weise gelang es 1929 Doisy und Butenandt gleichzeitig und unabhängig voneinander, als erstes Steroidhormon *Oestron* in kristallisierter Form zu isolieren. Schon drei Jahre später wurde durch Butenandt auch dessen Konstitution aufgeklärt.

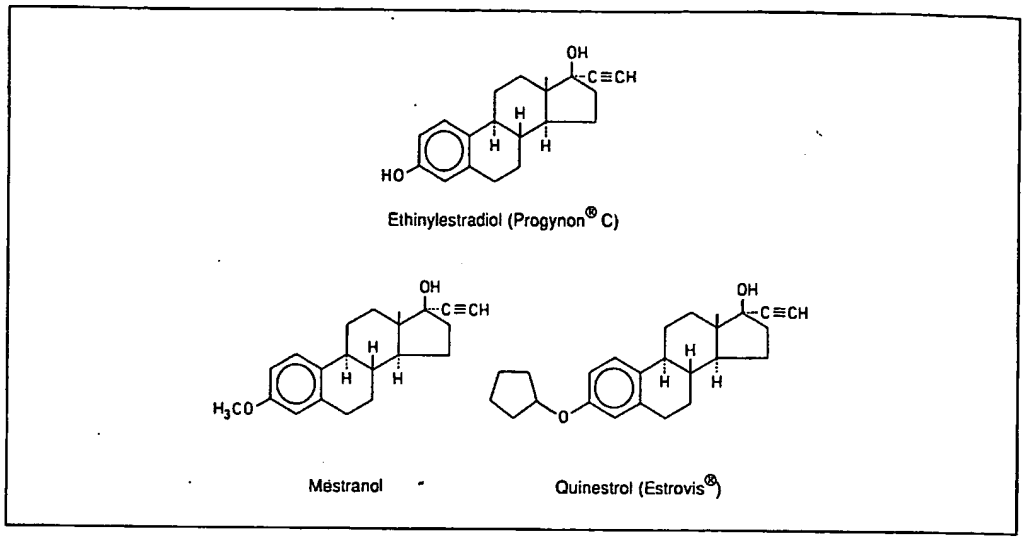
Das wichtigste in den Follikelepithelien produzierte oestrogene Hormon ist **Oestradiol** (Estradiol). Daneben wird *Oestron* (Estron) gebildet.

Oestrogenwirkungen. *Oestrogene, also vor allem Oestradiol, sind in der Hauptsache Wuchsstoffe, die auf die Geschlechtsorgane einwirken.* Sie *fördern* das *Wachstum der weiblichen Sexualorgane* und *prägen* die *sekundären weiblichen Geschlechtsmerkmale.* In der Pubertät bewirken sie den Schluß der Epiphysenfugen im Knochen und damit die *Beendigung des Längenwachstums.* Unter dem Einfluß der Oestrogene findet auch der *Aufbau der Uterusschleimhaut* und die *Bildung der Endometriumdrüsen* in der Proliferationsphase statt. Die *Viskosität des Zervikalsekrets* wird *erniedrigt. Für* das *Sexualverhalten* der Frau scheinen die Oestrogene *von untergeordneter Bedeutung* zu sein.

Oestrogene besitzen außerdem eine *schwache anabole Wirkung* und *vergrößern die subkutanen Fettdepots. Wachstum und Talgproduktion der Talgdrüsen* werden *verringert.* Oestrogene *steigern* ferner die *Calcium-Resorption* und die *Einlagerung von Calcium in den Knochen.* Der periphere Gefäßwiderstand in den kleinen Gefäßen wird verringert. Insbesondere in höheren Dosen führen Oestrogene zu einer *Retention von Natriumchlorid und Wasser.*

Biosynthese. Oestrogene werden aus Androgenen, insbesondere aus Testosteron und Androstendion (s. S. 335 f.) gebildet. Ein wichtiger Schritt hierbei ist die oxidative Entfernung der C-19-Methylgruppe durch einen Multienzymkomplex mit Aromatisierung des Ringes A. Bei Frauen im gebärfähigen Alter beträgt die Oestrogensekretion – abhängig von der Zyklus-





phase – 25 bis 100 µg pro Tag. Im Klimakterium fällt die Oestrogen-Sekretionsrate auf 5–10 µg täglich.

Kinetik. (Injiziertes) Oestradiol besitzt eine *Plasmahalbwertszeit* von etwa 50 Minuten. Oestradiol und Oestron unterliegen vor allem in der Leber einem vielfältigen Metabolismus durch Hydroxylierungen und Dehydrierungen sowie Konjugationen mit aktivierter Glucuronsäure sowie aktivem Sulfat. (Der First-pass-Effekt von oral appliziertem Oestradiol beträgt über 90%!) Ein Metabolit, der noch therapeutisch genutzt werden kann und vor allem *lokal* bei Veränderungen im Genitalbereich eingesetzt wird, ist *Oestriol* (Estriol; OeKolp®, Ortho-Gynest®, Ovestin®). Bei Frauen im Klimakterium werden außerdem *konjugierte Oestrogene,* die bei der Magen-Darm-Passage im Dickdarm dekonjugiert werden, verwendet.

Depot- und oral wirksame Oestrogene. Wegen der nur kurzen Wirkungsdauer bei parenteraler Applikation und der geringen Wirksamkeit nach oraler Gabe wurden Oestradiol-Derivate entwickelt, die länger bzw. besser oral wirksam sind.

Eine längere Wirkungsdauer besitzen *Oestradiol-Ester* (Tab. B 2-9), die nach intramuskulärer Injektion langsamer resorbiert und abgebaut werden.

Estradiol-valerat wird außerdem oral angewandt.

Wird an C-17 eine Ethinylgruppe eingeführt, erhält man *Ethinylestradiol,* das in der Leber nur langsam inaktiviert wird und daher oral gut wirksam ist. Ethinylestradiol und sein 3-Methylether *(Mestranol)* gehören zu den am häufigsten verwendeten Oestrogenen. Ein Analogpräparat ist *Quinestrol* (Estrovis®).

Oral wirksam sind auch totalsynthetisch gewonnene Oestrogene, z. B. Diethylstilbestrol, die kein Steroidgerüst mehr besitzen und nur noch entfernt mit den natürlichen Hormonen verwandt sind. Sie weisen ähnliche pharmakologische Eigenschaften wie Oestradiol auf. Da jedoch in einer Reihe von Fällen *Genitalkarzinome* bei Frauen beobachtet wurden, deren Mütter zur Erhaltung der Schwangerschaft bei drohendem Abort hochdosiert und über längere Zeit Diethylstilbestrol erhalten hatten, wurden die meisten Diethylstilbestrol enthaltenden Präparate aus dem Handel gezogen. Nur *Diethylstilbesterol-diphosphat* (Fosfestrol, s. S. 678) wird noch zur Behandlung des Prostatakarzinoms verwendet.

Indikationen. Oestrogene sind indiziert bei

- *Hypoplasie des Uterus und dessen Folgeerscheinungen* (z. B. Dysmenorrhoe),
- *allen Formen der Ovarialinsuffizienz,* vor allem nach operativer oder Röntgenkastration,

Tab. B 2-9. Oestradiol-Ester

Chemische Kurzbezeichnung	Handelspräparat (Eingetragenes Warenzeichen)
Estradiol-benzoat	Progynon B oleosum
Estradiol-valerat	Progynon Depot, Progynova
Estradiol-undecylat	Progynon Depot (zur Therapie des Prostatakarzinoms)

- *Oestrogenmangel im Klimakterium* (s.o.),
- *primärer und sekundärer Amenorrhoe* in zyklusgerechter Anwendung und in Kombination mit Gestagenen, ferner
- zum *primären* (Laktationshemmung) und *sekundären* (Laktationsunterdrückung) *Abstillen*.

Bedeutung hat die Oestrogenbehandlung auch beim *Prostatakarzinom* sowie beim *Mammakarzinom nach der Menopause* erlangt.

Dosierung. Die Dosierung erfolgt individuell und abhängig von der Indikation.

Zur Oestrogensubstitution, z.B. im Klimakterium oder nach Ovarektomie, kann Oestradiol nicht nur oral (z.B. Oestradiol-valerat 1–2 mg täglich), sondern auch – wegen Umgehung des First-pass-Effektes – in sehr viel niedrigerer Dosierung (25–100 µg pro Tag) *transdermal* appliziert werden.

Obwohl zahlreiche Oestrogenmangel-bedingte Beschwerden, z.B. Hitzewallungen, durch beide Applikationsarten gebessert werden können, ist bei transdermaler Anwendung im Gegensatz zur oralen Gabe die Osteoporose-prophylaktische Wirkung noch nicht gesichert.

Nebenwirkungen. *Oestrogene erhöhen das Thromboembolie-Risiko.* Bei Phlebitiden oder erhöhter Thrombosegefahr sind sie daher sofort abzusetzen. Bei langdauernden Oestrogengaben atrophieren ferner die Ovarien infolge einer Hemmung der Gonadotropinausschüttung. Spannungsgefühl in den Brüsten, Gewichtszunahme, Übelkeit, Natriumretention mit Ödembildung sowie Hyperpigmentierung der Haut können als weitere Nebenwirkungen hinzukommen.

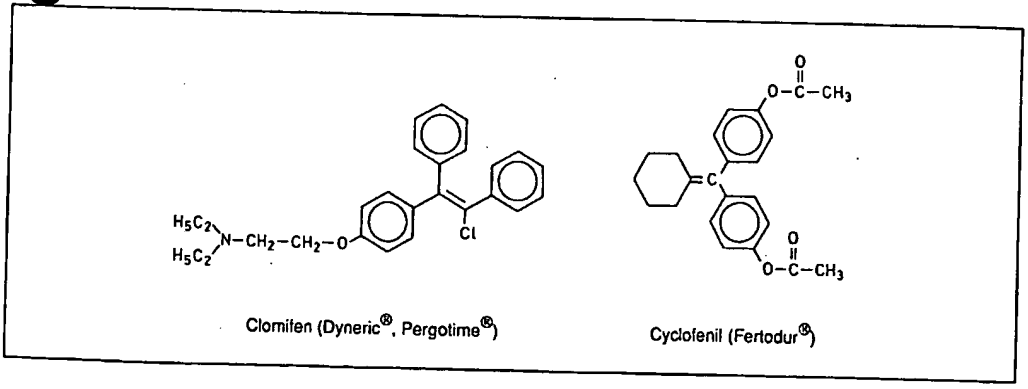
Kontraindikationen. Oestrogene sind kontraindiziert bei hormonabhängigen Uterus- und Mammatumoren, Endometriose, schweren Leberfunktionsstörungen, idiopathischem Schwangerschaftsikterus und schwerem Schwangerschaftspruritus in der Anamnese, Dubin-Johnson- und Rotor-Syndrom, thromboembolischen Erkrankungen sowie Sichelzellenanämie.

Interaktionen. Durch Enzyminduktoren, insbesondere Barbiturate, Carbamazepin oder Rifampicin, wird die Oestrogen-Wirkung herabgesetzt. Bei gleichzeitiger Gabe von Oestrogenen und Antidiabetika ist die Kohlenhydrat-Toleranz vermindert.

2.8.1.5 Antioestrogene

Antioestrogene sind Substanzen, die Oestrogenwirkungen ganz oder teilweise aufzuheben vermögen. Meist handelt es sich um *partielle* Antagonisten, d.h. um Stoffe mit einer schwachen agonistischen (oestrogenen) Wirkungskomponente. Hierzu gehören das Stilbenderivat *Clomifen* (Dyneric®, Pergotime®) und das Diphenylmethanderivat *Cyclofenil* (Fertodur®). Die beiden Stoffe führen beim Menschen zu einer vermehrten Freisetzung von Gonadoliberin und damit zu einer erhöhten Gonadotropinausschüttung. Sie werden daher bei Patientinnen mit anovulatorischen Zyklen zur Ovulationsauslösung verwendet. Ein *Erfolg* ist *nur bei* der *normogonadotropen Ovarialinsuffizienz*, d.h. bei intakter Hypothalamus-Hypophysen-Funktion zu erwarten. Als *Wirkungsmechanismus* wurde ermittelt, daß die beiden Substanzen den negativen Rückkopplungseffekt der Oestrogene im Hypothalamus durch Blockade der Rezeptoren aufheben. (Bei Frauen ohne Ovulation werden z.T. Oestrogene *kontinuierlich* und nicht wie üblich zyklisch gebildet.)

Die *Dosierung* von Clomifen beträgt 50 mg, von Cyclofenil 600 mg täglich für die Dauer von 5 Tagen. Die Anwendung hat unter ständiger ärztlicher Kontrolle zu erfolgen.



Clomifen (Dyneric®, Pergotime®) Cyclofenil (Fertodur®)

D7

REALLEXIKON DER MEDIZIN

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5. BAND

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Abbildungen, die nach Abbildungen anderer Veröffentlichungen bearbeitet wurden, werden in einer Aufstellung ausgewiesen, die der Schlußlieferung eines jeden Bandes beigefügt wird.

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e: oestrogenanaemia.

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e: oestrone unit.

Östron-Gruppe: Östrogene.

Östronschwefelsäure: im Körper als lösl. Ausscheidungsform gebildeter Östronester; s.a. gepaarte Säuren, Ätherglukuron- u. Ätherschwefelsäuren.

Östron-sulfotransferase; (EC 2.8.2.4 3'-Phosphoadenylylsulphate: oestrone sulphotransferase): Hydrolase, die die Reaktion Östron ⇆ Östron-3-sulfat katalysiert.

Oestrum: Oestrus (1).

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⟨–; *gr* mania ⇨ Wut, Wahnsinn⟩ – *e*: oestromania; erotomania; *f*: (2) estromanie (f.).

Östron, Estronum, Theelin, Folliculin: (1929/32) wichtigstes natürl. Follikelhormon (i.e.S.); 3-Hydroxy-1,3,5(10)-östratrien-17-on; in der Ther. weitgehend durch andere Östrogene (v.a. Östradiol) abgelöst.

Östron-Einheit: 0,1 g Östron (= 1 IE) als 1932 festgelegter östrogener Standard für biol. Vergleich im Tierversuch (anstelle der obsoleten Mäuse- u. Ratteneinheit) Heute ohne prakt. Bedeutung.
e: oestrone unit.

Östron-Gruppe: Östrogene.

Östronschwefelsäure: im Körper als lösl. Ausscheidungsform gebildeter Östronester; s.a. gepaarte Säuren, Ätherglukuron- u. Ätherschwefelsäuren.

Östron-sulfotransferase; (EC 2.8.2.4 3'-Phosphoadenylylsulphate: oestrone sulphotransferase): Hydrolase, die die Reaktion Östron ⇋ Östron-3-sulfat katalysiert.

Oestrum: Oestrus (1).

Soya – a dietary source of the non-steroidal oestrogen equol in man and animals

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ABSTRACT

The dietary origin of the weak oestrogen equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) present in human urine has been investigated using gas chromatography–mass spectrometry. Feeding experiments with different food constituents and monitoring the urinary excretion of equol revealed that soya food yields more than 0·1 mg urinary equol/g flour ingested. From this source the glucoside of daidzein (4',7-dihydroxyisoflavone) has been isolated and identified as a precursor of equol. Both equol and daidzein were characterized as monoglucuronide conjugates in human urine and the concentration of urinary equol exceeded the concentrations of the classical oestrogens by 100- to 1000-fold after ingestion of a single meal containing soya protein. The potential biological significance of this result is discussed.
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INTRODUCTION

Equol (7-hydroxy-3-(4'-(hydroxyphenyl)-chroman) is an isoflavan possessing weak oestrogenic activity, in the order of 10^{-3} times that of oestradiol (Braden, Hart & Lamberton, 1967; Shutt & Braden, 1968), while also being an antioestrogen in binding competitively with oestradiol to uterine cytosol receptors (Shutt & Cox, 1972; Tang & Adams, 1980). The compound was first discovered in the urine of pregnant mares over half a century ago (Marrian & Haslewood, 1932) and then in goat (Klyne & Wright, 1957), cow (Klyne & Wright, 1959), hen (MacRae, Dale & Common, 1960; Common & Aimsworth, 1961), sheep (Braden *et al.* 1967; Shutt & Braden, 1968) and rat (Axelson & Setchell, 1981). In these animals equol is formed by intestinal bacterial degradation of phyto-oestrogens present in the feed (Batterham, Hart, Lamberton & Braden, 1965; Nilsson, Hill & Davies, 1967; Shutt & Braden, 1968; Batterham, Shutt, Hart *et al.* 1971; Axelson & Setchell, 1981). Ingestion of larger quantities of clover, particularly *Trifolium subterraneum*, which has a high content of equol precursors, leads to an infertility syndrome in sheep referred to as clover disease, in which a cystic condition in the reproductive tract is accompanied by a failure to conceive (Bennetts, Underwood & Shier, 1946; Moule, Braden & Lamond, 1963; Morley, Axelsen & Bennett, 1964; Shutt, 1976).

The occurrence of equol in the urine of man was only recently reported (Axelson, Kirk, Farrant *et al.* 1982). The amounts excreted were similar to the endogenous oestrogens, but were not related to any hormonal status.

The dietary origin of equol in man is not known, but here we report that soya protein has a remarkably high content of an equol precursor which has been isolated and identified as the glucoside of the isoflavone, daidzein (4',7-dihydroxyisoflavone). This phyto-oestrogen is converted into equol, conjugated with glucuronic acid and is then excreted in urine.

MATERIALS AND METHODS

Urine samples

Urine (24 h) collections were obtained from a healthy man (age 34 years) and woman (age 25 years) and from 20 mature female rats (~200 g) of the Sprague–Dawley strain. The urine was collected in

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polyethylene flasks, frozen immediately and stored at −20 °C until analysed.

Diets

Rats were fed commercial pelleted food (Astra-Ewos, Södertälje, Sweden) or a semisynthetic diet, D7 (Midtvedt & Gustafsson, 1981), composed of wheat starch, casein, arachis oil, salts and vitamins. Food constituents (2–5 g/24 h) and equivalent amounts of extracts were tested for the presence of equol precursors by adding them to the semisynthetic diet in exchange for starch or oil and feeding it to one to five rats for 2 days. Soya flour (Soyolk; Soya Foods Ltd) was obtained from A/B Risenta, Stockholm, Sweden (composition: 40% protein, 20% fat and 20% carbohydrate). The two human subjects were given lunch meals in which 40 g of commercial textured soya (Natural Protoveg; Direct Foods Ltd, Petersfield, Hants; composition: 52% protein, 1% fat and 31·5% carbohydrate) was substituted for meat over a 5-day period. The soya was cooked according to the manufacturer's instructions.

Isolation of daidzein from soya

Soya flour (5 g) was refluxed for 1 h with 125 ml 80% aqueous ethanol. After filtration and evaporation of the alcohol, non-polar lipids were removed by first washing the aqueous extract with hexane (30 ml) and then passing it through a column bed (4 × 0·8 cm) of Lipidex 1000 (Packard Instrument Co., Downers Grove, Illinois, U.S.A.) (Dyfverman & Sjövall, 1978) in water. The aqueous effluent (about 30 ml) was then extracted with a Sep-Pak C_{18} cartridge (Waters Associates Inc., Milford, Maryland, U.S.A.) which was washed with 10 ml water before elution with 8 ml methanol (Shackleton & Whitney, 1980). The eluate was passed through a column bed (4 × 0·4 cm) of the strong cation exchanger sulphohydroxypropyl Sephadex LH-20 (SP-LH-20, H^+) (Axelson & Sjövall, 1979) and the material in the methanolic effluent (13 ml) was separated into neutral and phenolic fractions on a column bed (4 × 0·4 cm) of the strong anion exchanger triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20, OH^-) (Axelson, Sahlberg & Sjövall, 1981). Phenolic compounds, eluted from the column with 8 ml CO_2-saturated methanol, were further fractionated by preparative thin-layer chromatography (TLC) on a Merck precoated plate (silica gel 60 F_{254}; 20 cm × 20 cm, Merck, Darmstadt, West Germany) with ethylene chloride : acetic acid : water (10 : 10 : 1, by vol.). Spots were located by inspection under a u.v. lamp at 254 nm or for analytical TLC by spraying with a solution of H_2SO_4 : ethanol (1 : 1, v/v) and heating at 80–100 °C. Major components were scraped from the plate, eluted and rechromatographed on TEAP-LH-20 for removal of TLC impurities. After hydrolysis with β-glucosidase (Emulsin; Sigma Chemical Co., St Louis, Missouri, U.S.A.; 25 units in 5 ml 0·1 M-acetate buffer, pH 5, at 37 °C for 24 h), deconjugated compounds were extracted using a Sep-Pak C_{18} cartridge as described above.

Extraction and isolation of equol from urine

Equol was extracted from urine (1–6 ml) with a Sep-Pak C_{18} cartridge (Shackleton & Whitney, 1980) and conjugates, eluted with 8 ml methanol, were hydrolysed with *Helix pomatia* juice (Reactifes IBF Soc. Chim., Pointet Girard, Villeneuve la Garenne, France; 30 000 Fishman units β-glucuronidase in 5 ml 0·2 M-acetate buffer, pH 4·5) at 62 °C for 1 h (Scholler, Métay, Herbin & Jayle, 1966). After extraction on a Sep-Pak C_{18} cartridge, liberated equol in 8 ml methanol was purified by passage through a column bed (4 × 0·4 cm) of SP-LH-20 (H^+) (Axelson & Sjövall, 1979) and chromatography on a column (4 × 0·4 cm) of TEAP-LH-20 (OH^-) (Axelson *et al.* 1981). A phenolic fraction was obtained by elution of the latter column with 8 ml methanol saturated with CO_2. After removal of the CO_2 by application of vacuum, water was added to give a final concentration of 72% methanol and the sample passed through a column bed (2 × 0·4 cm) of diethylaminoethyl (DEAE)–Sephadex (Pharmacia, Uppsala, Sweden) in base form (Axelson *et al.* 1982). Monophenolic compounds were eluted with 10 ml 72% aqueous methanol and equol and other diphenolic compounds with 5 ml 72% aqueous methanol saturated with CO_2.

Isolation of equol and daidzein glucuronides from human urine

Urine (20 ml) collected from a male subject after 2 days on a soya diet was extracted on a Sep-Pak C_{18} cartridge, filtered through a column bed (4 × 0·4 cm) of SP-LH-20 (H^+) as described above and fractionated on a column bed (6 × 0·4 cm) of TEAP-LH-20 in OH^- form. After elution of monoglucuronides of neutral compounds with 20 ml 0·8 M-acetic acid in 72% aqueous methanol, glucuronide conjugates possessing a free phenolic group were eluted with 15 ml 0·4 M-formic acid in 72% methanol (Sahlberg, Axelson, Collins & Sjövall, 1981). An aliquot of the material in this fraction was analysed by TLC as for soya. R_F values of the glucuronides of equol and daidzein were 0·28 and 0·23 respectively (for comparison, oestriol 16α-glucuronide had an R_F value of 0·29). The carboxyl group of the glucuronic acid was methylated with diazomethane and the methyl esters were dissolved in methanol and purified on a column bed (4 × 0·4 cm) of TEAP-LH-20. After washing with 5 ml

methanol the conjugated equol and daidzein derivatives (which possess a free phenolic group) were eluted with 8 ml CO_2-saturated methanol.

Preparation of derivatives for gas chromatography (GC)–mass spectrometry (MS)

Methyl esters

The dried extract was dissolved in 1 ml diethyl ether : methanol (9 : 1, v/v) and diazomethane (freshly prepared by the reaction between N-methyl-N-nitroso-toluenesulphonamide and aqueous KOH, in diethyl ether; Schlenk & Gellerman, 1960) was added to the sample through a stream of nitrogen. After 30 min in an ice bath, excess diazomethane and solvents were removed under a stream of nitrogen.

Trimethylsilyl (TMS) ethers

Trimethylsilyl ethers were prepared by addition of 100 µl pyridine : hexamethyldisilazane : trimethylchlorosilane (3 : 2 : 1, by vol.) and heating at 60 °C for 30 min. The reagents were removed under a stream of nitrogen and the derivatives dissolved in hexane.

Deuterium-labelled TMS ethers were prepared by reaction with 100 µl [$^2H_9$]trimethylchlorosilane (Merck Sharp & Dohme Canada Ltd, Montreal, Canada) : pyridine (1 : 18, v/v) at 20 °C for 30 min.

Gas chromatography and mass spectrometry

Gas chromatography

Gas chromatography was carried out on a Pye 104 gas chromatograph equipped with a flame ionization detector and housing a 20 m × 0·3 mm open-tubular glass capillary column coated with SE-30 (Orion Analytica, Espoo, Finland). Nitrogen was the carrier gas with an inlet pressure of 50 kPa, giving a flow rate of about 1 ml/min. The oven temperature was 250 °C.

Quantification of equol present in the hydrolysed urine fractions was obtained by comparison of its GC peak area with that given by a known amount of authentic equol, having a retention time as a TMS ether derivative of 0·45 relative to that of 5α-cholestane. The limit of detection corresponded to about 1 µg equol in a 24-h urine collection.

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry was done on a modified LKB 9000 instrument housing an open-tubular glass capillary column (25 m × 0·3 mm) coated with SE-30, heated at 250 °C and connected to the ion source by a single stage adjustable jet separator (Axelson & Sjövall, 1977).

Derivatized conjugated compounds were analysed on a 1·5% SE-30 packed column (1 m × 3·4 mm) at 250 °C. Temperatures of the molecular separator and the ion source were 275 and 290 °C respectively; energy of bombarding electrons, 22·5 eV, ionizing current, 60 µA and accelerating voltage, 3·5 kV. Repetitive magnetic scanning (usually six to ten scans/min) over the range of mass/charge ratios (*m/z*) 0–800 daltons per unit electronic charge was initiated after a suitable delay from the time of sample injection. Methods for the computerized evaluation of the mass spectral data have been described (Axelson, Cronholm, Curstedt *et al.* 1974).

RESULTS

Dietary origin of equol

Since rats excrete equol in urine and bile (Axelson & Setchell, 1981) this animal was used as a model for man in the experiments screening for dietary precursors. Evidence that commercial pelleted food contains precursors of equol was obtained by changing a normal rat feed to that of a semisynthetic diet. A marked and rapid decrease in the urinary excretion of equol to less than 2 µg/24 h was followed by an increase after the diet reverted to pellets (Fig. 1). In the subsequent search for equol precursors, different food constituents and food extracts were added to this semisynthetic diet and the excretion of equol in urine was monitored. Of the variety of food-stuffs tested, which included soya flour, soya oil, wheat, rye, oat, millet, barley, buckwheat, corn, alfalfa, white beans and brown beans, soya flour provided the richest source of precursor(s) and resulted in the urinary excretion of about

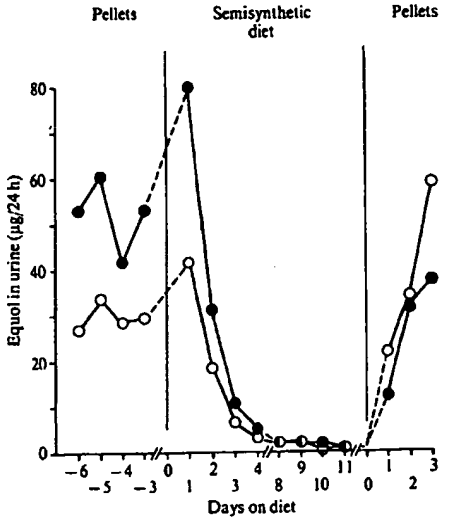

FIGURE 1. Daily urinary excretion of equol by two female rats fed commercial pelleted food or semisynthetic diet.

100 μg equol/g flour ingested (range 60–130 μg/g in five rats). The corresponding value for soya oil was only 5 μg/g. Little or no increase in the urinary excretion of equol (< 1 μg/g) was observed with the other food constituents tested. Commercial food pellets contain a proportion of soya cake sufficient to account for the urinary excretion of equol by rats.

Extending these studies to man, the urinary excretion of equol in two subjects (male and female), which is normally in the range of the classical oestrogens (Adlercreutz, Fotsis, Heikkinen *et al.* 1982; Axelson *et al.* 1982), increased 100- to 1000-fold to about 4–6 mg/24 h after ingestion of 40 g soya/day (Fig. 2). Thus the response to this diet was analogous to that observed in rats, indicating that the precursor–product relationships are similar in man and rats.

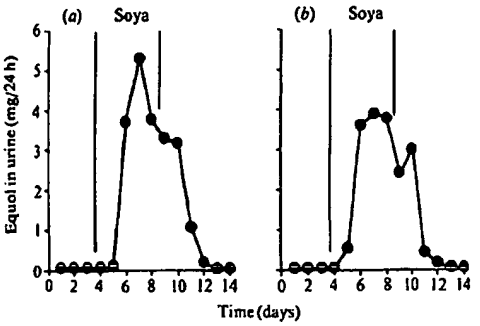

FIGURE 2. Daily urinary excretion of equol by (*a*) a man and (*b*) a woman before and after the addition of textured soya (40 g/day) to the normal diet.

Identification of daidzein, a precursor of equol in soya

Experiments to isolate the precursor(s) from soya flour showed that the compound(s) was extractable with 80% aqueous ethanol. After the fractionation of the extracted material into neutral and phenolic compounds on the ion exchanger TEAP-LH-20, approximately 90% of the precursor(s) was present in the phenolic fraction. Preparative TLC showed a major component of R_F 0·32. When this component was fed to rats it was readily converted to equol and quantitatively accounted for approximately 75% of the expected excretion of equol from the original amount of soya. The TMS ether of this compound, when analysed by GC–MS, did not show the molecular ion (M) at *m/z* 776 (Fig. 3) but ions at *m/z* 761 (M-15), 671 (M-(15+90)) and 581 (M-(15+2×90)), which are formed by loss of a methyl group from the molecule and subsequent loss of one and two derivatized hydroxyl groups respectively. The base peak at *m/z* 361 and the ions of *m/z* 271, 243, 217 and 204 are indicative of a TMS ether of a hexose structure (Laine & Elbein, 1971). In analogy with persilylated glucuronide conjugates (Spiegelhalder, Röhle, Sickmann & Breuer, 1976) the ion at *m/z* 450 indicated that the sugar residue was conjugated to an aromatic hydroxyl group. This ion arises from the glycone with a loss of a proton. Aromatic conjugation was further supported by the intense ion at *m/z* 398 (M-378), which is formed by deconjugation and transfer of a TMS group from the glycoside moiety to the aglycone (Billets, Lietman & Fenselau, 1973; Spiegelhalder *et al.* 1976). The ion at *m/z* 383 is formed by subsequent loss of a methyl group from the aglycone.

Treatment of this compound with β-glucosidase yielded a product (R_F value 0·83 compared with 0·92 for equol on TLC), the TMS ether derivative of which had an identical GC retention time on SE-30 (1·12 relative to 5α-cholestane) and mass spectrum as the persilylated authentic daidzein. The mass spectrum showed a molecular ion and base peak at *m/z* 398 (Fig. 3). The origin of ion *m/z* 355 is unknown, it may be due to loss of CH_3 (*m/z* 383) and CO (Budzikiewicz, Djerassi & Williams, 1964). The fragment ion at *m/z* 190 may represent $((CH_3)_3SiO\text{-}C_6H_4\text{-}C{\equiv}CH)^+$ in analogy with the fragmentation of equol (Axelson *et al.* 1982). These interpretations were supported by the analysis of the compound as a perdeuterated derivative.

The glycoside moiety of the daidzein conjugate occurring in soya was tentatively identified as a glucose residue. This is based on the following properties and evidence: hydrolysis with β-glucosidase, mobilities on the anion exchanger TEAP-LH-20, TLC and GC (retention time on SE-30 was 19 times that of the TMS ether of daidzein), the mass spectrum and the previous finding of daidzein glucoside in soya beans (Walz, 1931). Definite confirmation that daidzein is the major precursor of equol in commercial soya flour was obtained by feeding a rat the reference compound daidzein (acetate, 400 μg) in the semisynthetic diet, which yielded approximately 50 μg equol in the urine.

Identification of equol and daidzein glucuronides in human urine

In our earlier characterization of equol in urine (Axelson *et al.* 1982) it was tentatively identified as a glucuronide conjugate, consistent with the majority of endogenous urinary steroids. After repetitive scanning MS of the GC-effluent, the presence of equol and daidzein glucuronides was evident from the reconstructed chromatograms for diagnostically significant ions given by the methyl ester TMS ether derivative of the two compounds (Fig. 4). Peaks occurred at 14 and 32 min (retention time of 5α-cholestane was about

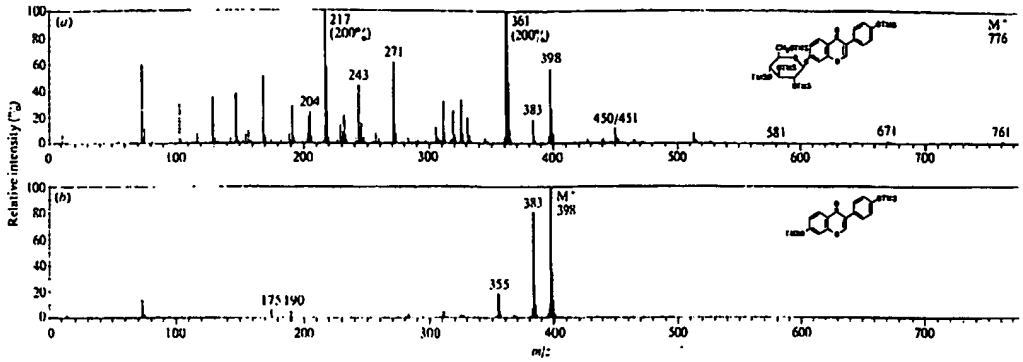

FIGURE 3. Electron impact ionization mass spectra of the trimethylsilyl (TMS) ethers of daidzein (4',7-dihydroxy-isoflavone) glucoside isolated from soya flour (*a*) before and (*b*) after treatment with β-glucosidase. M^+, molecular ion; *m/z*, mass/charge ratio in daltons per unit electronic charge.

1 min) which represent intact glucuronides of equol and daidzein respectively. The complete mass spectrum of equol glucuronide eluted at 14 min is shown in Fig. 5. Consistent with the mass spectra of derivatives of glucuronide conjugates of oestrogens (Spiegelhalder *et al.* 1976) the relative intensities of the molecular ion (*m/z* 720) and the ions formed by losses of a methyl group (*m/z* 705) and TMS groups (*m/z* 615 and 525) are below 5% in this aromatic conjugate. The loss of 292 and 334 mass units from the molecular ion has previously been observed in mass spectra of phenolic glucuronide conjugates (Billets *et al.* 1973; Spiegelhalder *et al.* 1976; Axelson & Setchell, 1980); the latter fragmentation represents the loss of glucuronic acid with the corresponding transfer of a TMS group to equol. The ion at *m/z* 192 consists of a derivatized phenol group with a 2-carbon chain which is the base peak in the mass spectrum of unconjugated equol (Axelson *et al.* 1982). Ions at *m/z* 406, 407, 317 (base peak), 275, 217 and 204 are all typical of the fragmentation of the glucuronic acid (Billets *et al.* 1973; Spiegelhalder *et al.* 1976; Axelson & Setchell, 1980).

The glucuronide conjugate of daidzein was also identified in the same urine sample (Fig. 4). The mass spectral fragmentation pattern of the methyl ester TMS ether of the intact conjugate (Fig. 5) was similar

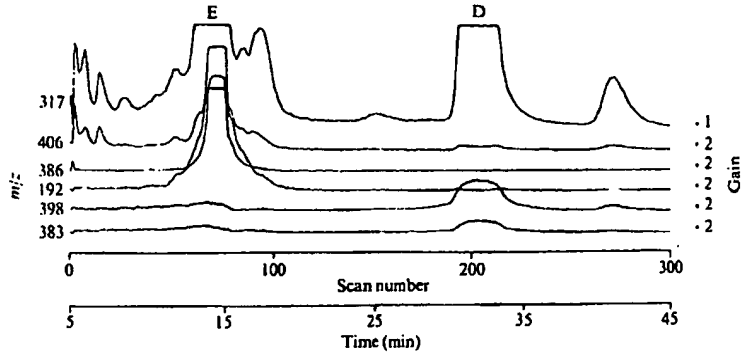

FIGURE 4. Gas chromatographic–mass spectrometric analysis of methyl ester trimethylsilyl ether derivatives of intact glucuronides of equol (E; 7-hydroxy-3-(4'-hydroxyphenyl)-chroman) and daidzein (D; 4',7-dihydroxy-isoflavone) isolated from human urine. Fragment ion current chromatograms of mass/charge ratio (*m/z*) 317 daltons per unit electronic charge and *m/z* 406 are representative of the glucuronyl moiety, those of *m/z* 386 and *m/z* 192 of equol and those of *m/z* 398 and *m/z* 383 of daidzein structure (see text). For purpose of illustration the intensities of *m/z* 317 were multiplied by a factor of 1, the other ions by a factor of 2.

to that of equol glucuronide. The loss of glucuronic acid in a rearrangement with the simultaneous transfer of a TMS group to the aglycone gives rise to the significant ion at *m/z* 398 (M-334) which is the molecular ion and base peak in the mass spectrum of the TMS ether of daidzein (Fig. 3).

These data firmly establish the occurrence of equol and daidzein in human urine as glucuronide conjugates. However, it is not possible to determine the position of conjugation by these methods, and the presence of two isomers of each compound cannot be excluded.

weeks the capacity to form equol decreased or disappeared in several rats. When pelleted food was again given, the capacity was partly regained. A variable yield of urinary equol has also been observed in man after ingestion of soya (Setchell *et al.* 1984). Conjugation of equol and daidzein with glucuronic acid most likely occurs in the liver as is the case with most endogenous oestrogens.

Although daidzein was the only equol precursor identified from soya, other precursors may exist in plants and foods. Isoflavones such as formononetin (7-hydroxy-4'-methoxyisoflavone), biochanin A (5,7-di-

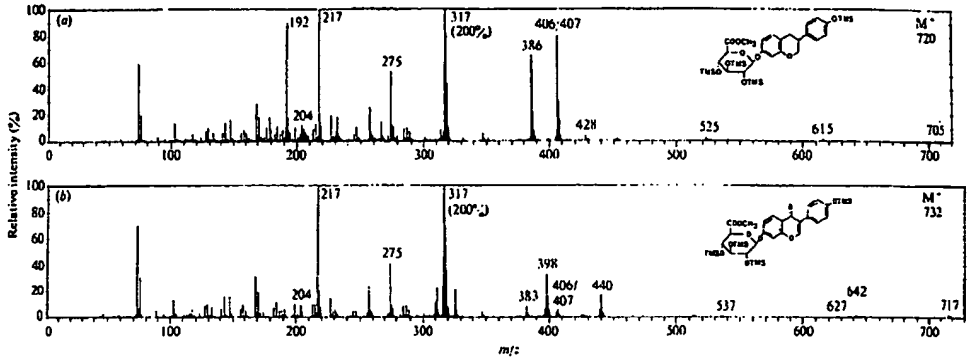

FIGURE 5. Mass spectra of the methyl ester trimethylsilyl (TMS) ether derivatives of the glucuronides of (*a*) equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) and (*b*) daidzein (4',7-dihydroxy-isoflavone) isolated from human urine. The origin of ions is given in the text. M^+, molecular ion; *m/z*, mass/charge ratio in daltons per unit electronic charge.

DISCUSSION

As shown here, and in a subsequent study (Setchell, Borriello, Hulme *et al.* 1984), soya meal is a major dietary source of urinary equol in man. Equol itself was not detected in soya but was shown to be formed from the glucoside of daidzein. Glucosidases are known to be present in intestinal bacteria (Drasar & Hill, 1974), which have also been shown to carry out the reduction and deoxygenation reactions required for conversion of daidzein to equol in animals (Batterham *et al.* 1965; Nilsson *et al.* 1967; Batterham *et al.* 1971) and recently in man (Setchell *et al.* 1984). Germfree rats, however, do not excrete equol when given commercial pelleted food (Axelson & Setchell, 1981). These results strongly suggest that also in man equol is formed in the gastrointestinal tract as a result of the bacterial degradation of daidzein. Thus, the rate of formation of equol from daidzein is conceivably influenced by the composition of the microflora, the intestinal transit time and the redox level in the large intestine. These conditions are affected by the diet, and when rats were fed the semisynthetic diet for several

hydroxy-4'-methoxyisoflavone) and genistein (4'-5,7-trihydroxyisoflavone) are all potential precursors of equol in animals (Cayen, Carter & Common, 1964; Batterham *et al.* 1965, 1971; Nilsson *et al.* 1967; Shutt & Braden, 1968; Tang & Common, 1968; Batterham *et al.* 1971). Soya beans can contain an abundance of phyto-oestrogens (Walz, 1931; Walter, 1941; Naim, Gestetner, Kirson *et al.* 1973; Lookhart, Jones & Finney, 1978), particularly genistein and daidzein, which have been ascribed to cause uterotrophic effects in laboratory mice given soya bean meal (Carter, Smart & Matrone, 1953; Cheng, Story, Yoder *et al.* 1953) or commercial pelleted food (Drane, Patterson, Roberts & Saba, 1975, 1980). Our observations here and earlier that equol is the major phenolic compound found in urine, blood and bile of rats maintained on this diet (Axelson & Setchell, 1981) suggest that the oestrogenic effects are more likely to be induced *in vivo* by equol than by genistein and/or daidzein. Genistein glucoside was not detected in the commercial soya flour used in the present study. Whether this is due to variations in the composition of isoflavones between different species of soya beans or due to elimination of

genistein during the manufacture of the flour is not known.

The widespread use of soya beans as a protein food source makes it important to determine possible physiological effects of equol in man. The 'contraceptive' effect in animals suggests to us that it may be of interest to investigate the dietary habits and urinary excretion of equol in women with unexplained infertility or disorders of the menstrual cycle.

In addition, whether the presence of phytoestrogens and related oestrogenic compounds in common food-stuffs affect the development and/or treatment of hormone-dependent tumours should also be considered.

ACKNOWLEDGEMENTS

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Catheterisation: your urethra in their hands

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The emphasis in undergraduate medical education is often on the theoretical aspects of medicine rather than the practical aspects. Practical procedures are commonly taught informally, the teaching being passed from one junior to the next.[1] The philosophy is of "See one, do one, teach one." Urethral catheterisation is a procedure that requires a certain amount of skill, knowledge, and experience and is not without complication,[2-4] yet it is usually delegated to the most junior and inexperienced medical staff, the junior house officers.

Subjects, methods, and results

To assess the level of competence at catheterisation among junior medical staff house officers at this hospital were interviewed with a structured questionnaire, covering three aspects of the procedure: the degree of undergraduate and postgraduate instruction, the practical and theoretical aspects of catheterisation, and, finally, problems and complications encountered.

Thirty junior house officers (graduates of five medical schools) were interviewed. Eighteen were male and 12 were female. The replies to the questionnaire showed that none of the interviewees had received any formal instruction regarding any aspect of urethral catheterisation as an undergraduate. Practical postgraduate instruction in 24 was limited to supervision of a single catheterisation, and four subjects were unsupervised. Although those interviewed had performed a mean of 28 (range 6-100) catheterisations in male patients, only four of them had catheterised female patients.

Despite the large number of procedures performed there was appreciable ignorance of the practical and theoretical aspects of catheterisation. Twenty five interviewees were unaware of the availability of short term and long term catheters or of the duration for which they may be safely left without being changed. Three interviewees simply used the catheter that was provided by the nursing staff, and one did not know that different sizes existed.

Twenty eight interviewees initially used force when meeting resistance to the passage of the catheter, and 13 stated that the development of fresh urethral bleeding would not deter them from a further attempt at catheterisation. Eighteen were happy to attempt catheterisation in a patient who had a known urethral stricture. Five interviewees were unaware of the difference between a phimosis and paraphimosis.

Despite the lack of formal tuition all had developed what seemed to be a satisfactory aseptic technique. None, however, was aware of the nature of the antiseptic fluid or the strength of the local anaesthetic gel, but simply used what was provided by the nursing staff.

Nineteen of the interviewees had encountered bleeding and six had had patients in whom a paraphimosis had developed after catheterisation. A particularly disturbing finding was that, although 14 interviewees had requested help from senior medical staff, seven were reluctant to seek advice, because of their impression that difficulties with catheterisation were not worthy of disturbing senior staff. Eight of the 12 female medical staff had encountered problems with male patients becoming sexually excited during the procedure.

Discussion

The results of our survey suggest that the technique of urethral catheterisation is poorly taught, and in the light of these results we are preparing a short teaching video to be shown to every house officer at the start of their preregistration post.

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Oestrogenic effects of plant foods in postmenopausal women

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Crops grown as animal pasture are known to have oestrogenic activity,[1] and some foods contain potential oestrogenic analogues such as the isoflavonoids (isoflavones and coumestans), lignans, and resorcyclic acid lactones,[2] which may be activated or inactivated.[3] We studied the effect of three foods reported to induce vaginal oestrus in laboratory animals[4] in postmenopausal women not taking oestrogen replacement therapy.

Subjects, methods, and results

We studied 25 postmenopausal women who were non-smokers, in good general health, and taking no drugs known to affect oestrogen state (mean age 59 (range 51-70); body mass index 24·4 (range 18·7-31·6) kg/m^2; years after menopause 8·1 (range 1-20)). The protocol was a latin square design with a two week run in period and a six week experimental period. The women recorded their normal diet for 14 days and were asked to repeat the fortnightly diet throughout the study. During the experimental period the diet was

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supplemented with soya flour (45 g daily), red clover sprouts (10 g dry seed daily), and linseed (25 g daily), each for two weeks in turn. To check compliance the women returned residual food. Blood samples were taken weekly and lateral wall vaginal smears taken fortnightly and at follow up two and eight weeks after supplementation finished. Analysis was on intention to treat, but 23 women completed the study.

We examined the dependent variables vaginal cell maturation and serum concentrations of luteinising hormone and follicle stimulating hormone. The cumulative effects of the three foods at six weeks were compared with baseline by the paired *t* test, as were the residual effects, two and eight weeks after the last food supplement. We found significant differences in vaginal cytology after six weeks' supplementation ($p<0.01$, 95% confidence interval 6·0 to 17·6), which persisted for two weeks after treatment ($p<0.02$), but cytology returned to baseline after eight weeks (table).

Mean (SE) values for oestrogenic indicators in postmenopausal women consuming phyto-oestrogens

Week		Maturation value	Luteinising hormone (IU/l)	Follicle stimulating hormone (IU/l)
1			45·7 (3·1)	58·7 (2·9)
2		30·8 (4·5)	46·6 (3·4)	58·7 (3·0)
3			50·8 (8·5)	57·4 (2·9)
4		35·0 (5·1)	46·0 (3·6)	57·3 (2·9)
5	Food		46·2 (3·3)	57·7 (3·0)
6	supplementation	39·6 (5·3)	42·9 (3·2)	54·3 (2·9)
7			43·6 (3·3)	56·4 (2·8)
8		43·4 (3·6)	44·6 (3·3)	56·6 (2·4)
9			44·9 (3·5)	57·9 (2·8)
10		43·6 (4·7)	44·9 (3·3)	57·5 (2·7)
16		33·7 (5·5)		

The maturation value significantly increased after soya flour ($p<0.05$) and linseed ($p<0.02$) but not after red clover sprouts ($p=0.11$).

All women had concentrations of follicle stimulating hormone and luteinising hormone greater than those in the premenopausal range of 2·8 IU/l and 6·13 IU/l respectively. There was a cumulative effect on serum concentrations of follicle stimulating hormone ($p<0.05$) but not on luteinising hormone over the six week supplementation period. Individual two week food supplements had no measurable effects on either hormone.

In seven women with the most pronounced changes in vaginal cytology we measured serum oestradiol concentrations weekly. Baseline concentrations were <70 pmol/l in all but one woman, who was retained as the study was based on intention to treat. There were no appreciable changes in body weight during the study.

Comment

We aimed to consider whether phyto-oestrogens were of consequence in human nutrition. Our study gives some indication of the recovery time from any possible effect of treatment and also provides further evidence of causality. Vaginal maturation is a sensitive and specific indicator of oestrogenicity. Follicle stimulating hormone is less sensitive to weak oestrogenic compounds such as phyto-oestrogens. Weak oestrogenic compounds may sometimes act as anti-oestrogens, which may affect their usefulness as sources of oestrogenic activity. Conversely, tamoxifen, an antioestrogen, can have oestrogenic effects on vaginal cytology.[5]

Patterns of food intake may modulate the severity of the menopause as it is an oestrogen deficiency state. Up to half of the diet of some populations may comprise foods containing phyto-oestrogens, whereas in our study such foods comprised only about 10% of energy intake for a fairly short time. Whether menopausal symptoms differ in such populations would be worth investigation.

We thank our statistical adviser, Steve Farrish, from the department of social and preventive medicine, Monash University.

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Inadvertent duplicate publication

Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears

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The *BMJ* regrets that much of the material in the above article (30 June 1990, p 1690) was substantially the same as that published previously in *Contemporary Reviews in Obstetrics and Gynaecology* (Redman CWE, Buxton EJ, Cullimore J, Luesley DM. Loop diathermy excision of the cervical transformation zone in the management of cervical intraepithelial neoplasia. 1990;2:53-8). The authors did not tell us this when the article was submitted, their article did not contain any reference to the earlier paper, and all authors signed our copyright form, which states, among other things, that "papers are accepted on condition that they have not been published by any other journal."

We regret this inadvertent duplicate publication, for which the authors hold sole responsibility, and which is in violation of our Instructions to Authors and internationally agreed guidelines.

Correction

Incidence of peptic ulcer disease in Gothenburg, 1985

An editorial error occurred in this paper by Dr Ivi-Mai Schöön and others (1989;299:1132). The y axis of figure 1 should read 0, 5, 10, 15, and 20 and not 0, 0·5, 1·0, 1·5, and 2·0 as published.

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D10

OBJECTIVE INDICATOR FOR THE ASSESSMENT OF POSTMENOPAUSAL HOT FLASHES

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Key words: Hot flashes • Skin temperature recording • Estrogen therapy • Chinese herbal medicine

Introduction

The most characteristic symptom in the climacterium is the so-called hot flashes which is described as a sudden feeling of warmth over the face, neck and upper thorax, followed by profuse sweating in the same areas, generally lasting a few minutes. The subjective nature of this disturbance has hampered the standardized evaluation of responses to various therapeutic agents. Recent studies by Molnar[4] and Meldrum et al.[3] have shown that the recording of skin temperature changes of the finger provides an objective index of hot flashes. The present study was undertaken to evaluate an objective method of monitoring postmenopausal hot flashes in order to select a patient for hormonal therapy. In addition, substitution of Chinese herbal medicine for estrogen was also assessed in this study because of risk of potentially dangerous side effects[1][2][5].

Patients and Method

Fifty patients were studied who were experiencing frequent severe hot flashes after surgical or natural menopause. Only those without other complicating illness were enrolled into the study. The subjects had not received estrogen therapy for at least 4 weeks before study. Skin temperature was recorded over the dorsum of the proximal phalanx of the third finger of the non-dominant hand using Terumo thermister probe and deep body temperature monitor. A continuous recording was obtained with Terumo recorder on a slow chart (30mm/h.). During the recording, the subjects were instructed to press a push-button on the onset of each subjective feeling of flushing so that a mark was recorded on a chart instantaneously. Then the patients completed Cornell Medical Index as a screening device for evaluating the emotional state. At the same time, venous blood samples were drawn for laboratory examinations. Each of 50 patients received Premarin (conjugated equine estrogens) 1.25mg daily for 2 weeks and among those the 40 patients were then treated with Chinese herbal medicine as an alternative to Premarin for recurring hot flashes following discontinuation of Premarin treatment. The Chinese herbal medicine used in the present study was supplied as dry granule of herb extracts by Tsumura Juntendo, Inc.. The patient's subjective opinion of treatment results was classed as much improved, improved, somewhat improved, unchanged or worse after two-week treatment.

Results

Through the study, the 50 patients were subdivided into three groups depending on the pattern of skin temperature changes.

The skin temperature always started to rise on each onset of subjective feeling of flushing. This pattern of temperature changes represents group 1 (Fig. 1).

Coincidence of temperature rise with flushing was noted only once through the recording. No other correlation was found between them. This pattern represents group 2 (Fig. 2).

No correlation between temperature rise and flushing was demonstrated through the recording, representing group 3 (Fig. 3).

The effect of Premarin on hot flashes was shown in table 1. In group 1, the response of the patients to Premarin was remarkable. In group 2, the effect was classed as somewhat improved because of insomnia, anxiety and so on. But they were successfully treated with tranquilizer combined with Premarin. In group 3, the patients failed to respond to Premarin and later in all cases, the diagnosis of neurosis was predicated by a psychiatrist.

The relationship between the mean levels of each hormone measured in each group and the response to Premarin treatment was found to be insignificant (Table 2).

Unlike modern medicine, in Chinese medicine, therapy is indicated for a symptom-complex rather than a disease. Therefore, patients suffering from the same disease may be given a different medicine; conversely, patients with different disease may receive the same formula. In this study, herbal formulas widely used for climacteric disorder were

Fig. 1.

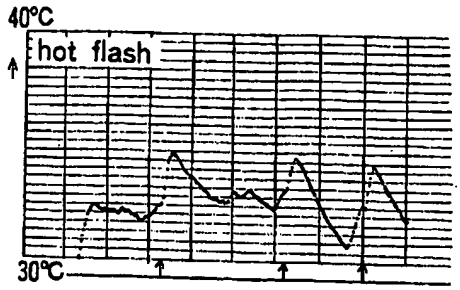
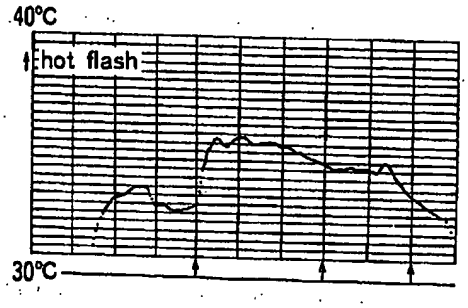


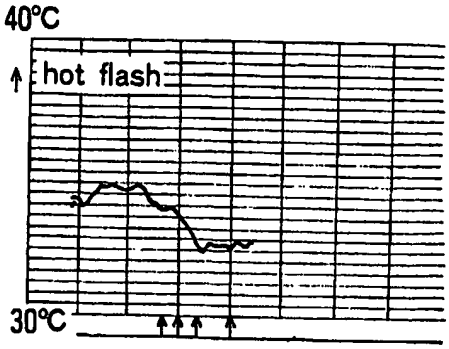
Fig. 2.



chosen as an alternative to Premarin and those used for nervousness were selected as an alternative to tranquilizers (Fig. 4).

As shown in table 3, a satisfactory response was obtained with Chinese herbal medicine in the patients of group 1 and 2 if hot flashes resulted from natural menopause, but no noticeable effect, rather discomfort was obtained if hot flashes resulted from surgical menopause. In group 3, herbal medicine was ineffective as was Premarin.

Fig. 3.



Discussion

In treating women with hot flashes, it is essential to differenciate the cause whether endocrinic or neurotic. In this study, neither hormonal analysis nor psychological testing was found to be effectual. On the other hand, skin temperature recording was shown to make it possible to evaluate hot flashes objectively and to select patients for whom Premarin treatment was expected to be effective without other screening aids.

Additionally, a guide-line of herbal formulas for hot flashes was suggested and no serious side effects have been noticed.

Our recommendation, therefore, would be that women with hot flashes should be classified depending on the pattern of skin temperature changes before prescription (Fig. 5). Where coincidence of temperature rise with flushing is noted, Chinese herbal formulas should be indicated as a first choice if hot flashes result from natural menopause,

Table 1. Effect of Premarin on hot flashes

Clinical group	No. patients	Skin temp. changes	CMI	Response to Premarin
1	30	+	I ~ III	much improved or improved
2	15	±	I ~ IV	somwhat improved
3	5	−	III ~ IV	unchanged

+ : The skin temperature always started to rise on each onset of subjective feeling of flushing.

± : Coincidence of temperature rise with flushing was occasionally noted.

− : No correlation between temperature rise and flushing was demonstrated.

Table 2. Concentrations of gonadotropins and steroids in each group

Clinical group	No. patients	FSH* (mIU/ml)	LH* (mIU/ml)	E_1* (pg/ml)	E_2* (pg/ml)	E_3* (pg/ml)
1	30	112.6± 7.9	131.1± 6.8	46.1± 3.5	23.8± 4.4	<5.00
2	15	85.8±10.1	143.7±20.6	75.8±16.9	60.5±23.8	<5.00
3	5	76.9±11.2	130.3±24.2	50.0±16.5	13.7± 3.7	<5.00

*Mean±SEM

Table 3. Effect of Chinese herbal medicine on hot flashes

Clinical group	Skin temp. changes	CMI	No. success/total (%)	
			Natural menopause	Surgical menopause
1	+	I ~ III	11/11(100)	3/19(16)
2	±	I ~ IV	3/3 (100)	1/3 (33)
3	−	III ~ IV	0/3 (0)	0/1 (0)

Fig. 4. Indication of Chinese herbal medicine for hot flashes.

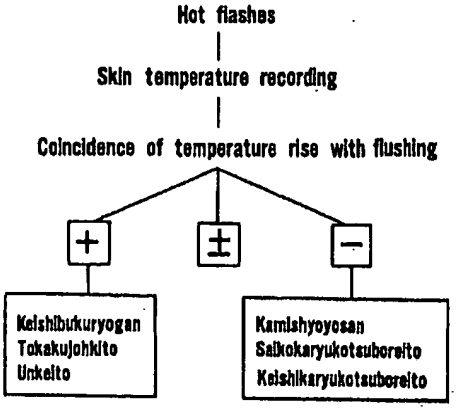
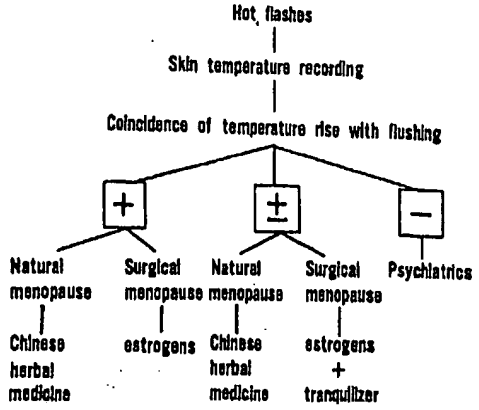


Fig. 5. Evaluation and treatment of women with hot flashes.



because of the potential risk of carcinogenesis of estrogens; on the other hand, estrogens can be prescribed liberally if hot flashes result from surgical menopause.

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Acknowledgement

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3. *Meldrum, D.R., Shamonki, I.M., Frumar, A.M., Talaryn, I.V., Chang, R.J. and Judd, H.L.:* Elevations in skin temperature of the finger as an objective index of postmenopausal hot flashes: Standardization of the technique. Am. J. Obstet. Gynecol., 135: 713, 1979.
4. *Molner, G.W.:* Body temperatures during menopausal hot flashes. J. Appl. Physiol., 38: 499, 1975.
5. National Institutes of Health Consensus Development Conference: Estrogen use in postmenopausal women. Ann. Intern. Med., 91: 921, 1979.

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OBJECTIVE INDICATOR FOR THE ASSESSMENT OF POSTMENOPAUSAL HOT FLASHES

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Department of Obstetrics and Gynecology, Hiratsuka City Hospital, Hiratsuka
(Director : Ryuichi Nakayama)

Key words: Hot flashes • Skin temperature recording • Estrogen therapy • Chinese herbal medicine

Introduction

The most characteristic symptom in the climacterium is the so-called hot flashes which is described as a sudden feeling of warmth over the face, neck and upper thorax, followed by profuse sweating in the same areas, generally lasting a few minutes. The subjective nature of this disturbance has hampered the standardized evaluation of responses to various therapeutic agents. Recent studies by Molnar[4] and Meldrum et al.[9] have shown that the recording of skin temperature changes of the finger provides an objective index of hot flashes. The present study was undertaken to evaluate an objective method of monitoring postmenopausal hot flashes in order to select a patient for hormonal therapy. In addition, substitution of Chinese herbal medicine for estrogen was also assessed in this study because of risk of potentially dangerous side effects[1)2)5)].

Patients and Method

Fifty patients were studied who were experiencing frequent severe hot flashes after surgical or natural menopause. Only those without other complicating illness were enrolled into the study. The subjects had not received estrogen therapy for at least 4 weeks before study. Skin temperature was recorded over the dorsum of the proximal phalanx of the third finger of the non-dominant hand using Terumo thermister probe and deep body temperature monitor. A continuous recording was obtained with Terumo recorder on a slow chart (30mm/h.). During the recording, the subjects were instructed to press a push-button on the onset of each subjective feeling of flushing so that a mark was recorded on a chart instantaneously. Then the patients completed Cornell Medical Index as a screening device for evaluating the emotional state. At the same time, venous blood samples were drawn for laboratory examinations. Each of 50 patients received Premarin (conjugated equine estrogens) 1.25mg daily for 2 weeks and among those the 40 patients were then treated with Chinese herbal medicine as an alternative to Premarin for recurring hot flashes following discontinuation of Premarin treatment. The Chinese herbal medicine used in the present study was supplied as dry granule of herb extracts by Tsumura Juntendo, Inc.. The patient's subjective opinion of treatment results was classed as much improved, improved, somewhat improved, unchanged or worse after two-week treatment.

Results

Through the study, the 50 patients were subdivided into three groups depending on the pattern of skin temperature changes.

The skin temperature always started to rise on each onset of subjective feeling of flushing. This pattern of temperature changes represents group 1 (Fig. 1).

Coincidence of temperature rise with flushing was noted only once through the recording. No other correlation was found between them. This pattern represents group 2 (Fig. 2).

No correlation between temperature rise and flushing was demonstrated through the recording, representing group 3 (Fig. 3).

The effect of Premarin on hot flashes was shown in table 1. In group 1, the response of the patients to Premarin was remarkable. In group 2, the effect was classed as somewhat improved because of insomnia, anxiety and so on. But they were successfully treated with tranquilizer combined with Premarin. In group 3, the patients failed to respond to Premarin and later in all cases, the diagnosis of neurosis was predicated by a psychiatrist.

The relationship between the mean levels of each hormone measured in each group and the response to Premarin treatment was found to be insignificant (Table 2).

Unlike modern medicine, in Chinese medicine, therapy is indicated for a symptom-complex rather than a disease. Therefore, patients suffering from the same disease may be given a different medicine; conversely, patients with different disease may receive the same formula. In this study, herbal formulas widely used for climacteric disorder were

Fig. 1.

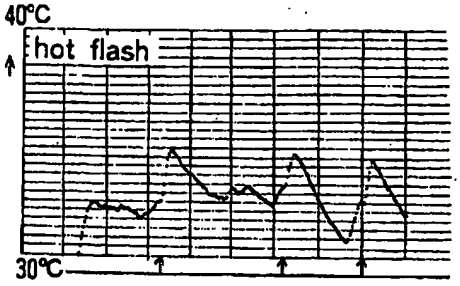
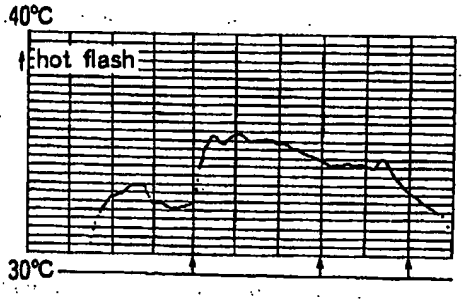


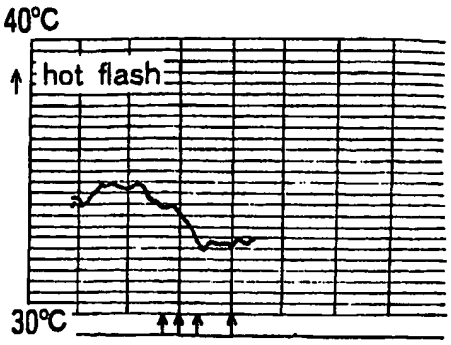
Fig. 2.



chosen as an alternative to Premarin and those used for nervousness were selected as an alternative to tranquilizers (Fig. 4).

As shown in table 3, a satisfactory response was obtained with Chinese herbal medicine in the patients of group 1 and 2 if hot flashes resulted from natural menopause, but no noticeable effect, rather discomfort was obtained if hot flashes resulted from surgical menopause. In group 3, herbal medicine was ineffective as was Premarin.

Fig. 3.



Discussion

In treating women with hot flashes, it is essential to differenciate the cause whether endocrinic or neurotic. In this study, neither hormonal analysis nor psychological testing was found to be effectual. On the other hand, skin temperature recording was shown to make it possible to evaluate hot flashes objectively and to select patients for whom Premarin treatment was expected to be effective without other screening aids.

Additionally, a guide-line of herbal formulas for hot flashes was suggested and no serious side effects have been noticed.

Our recommendation, therefore, would be that women with hot flashes should be classified depending on the pattern of skin temperature changes before prescription (Fig. 5). Where coincidence of temperature rise with flushing is noted, Chinese herbal formulas should be indicated as a first choice if hot flashes result from natural menopause,

Table 1. Effect of Premarin on hot flashes

Clinical group	No. patients	Skin temp. changes	CMI	Response to Premarin
1	30	+	I ~ III	much improved or improved
2	15	±	I ~ IV	somwhat improved
3	5	−	III ~ IV	unchanged

+ : The skin temperature always started to rise on each onset of subjective feeling of flushing.

± : Coincidence of temperature rise with flushing was occasionally noted.

− : No correlation between temperature rise and flushing was demonstrated.

Table 2. Concentrations of gonadotropins and steroids in each group

Clinical group	No. patients	FSH* (mIU/ml)	LH* (mIU/ml)	E_1* (pg/ml)	E_2* (pg/ml)	E_3* (pg/ml)
1	30	112.6± 7.9	131.1± 6.8	46.1± 3.5	23.8± 4.4	<5.00
2	15	85.8±10.1	143.7±20.6	75.8±16.9	60.5±23.8	<5.00
3	5	76.9±11.2	130.3±24.2	50.0±16.5	13.7± 3.7	<5.00

* Mean±SEM

Table 3. Effect of Chinese herbal medicine on hot flashes

Clinical group	Skin temp. changes	CMI	No. success/total (%)	
			Natural menopause	Surgical menopause
1	+	I～III	11/11(100)	3/19(16)
2	±	I～IV	3/3 (100)	1/3 (33)
3	−	III～IV	0/3 (0)	0/1 (0)

Fig. 4. Indication of Chinese herbal medicine for hot flashes.

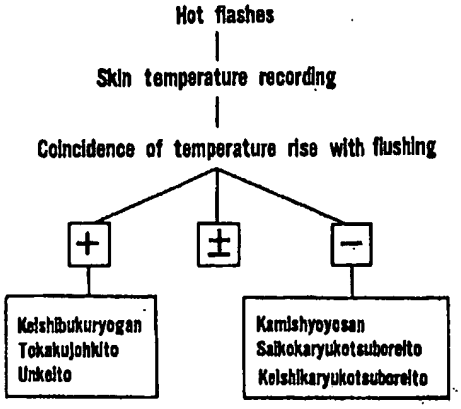
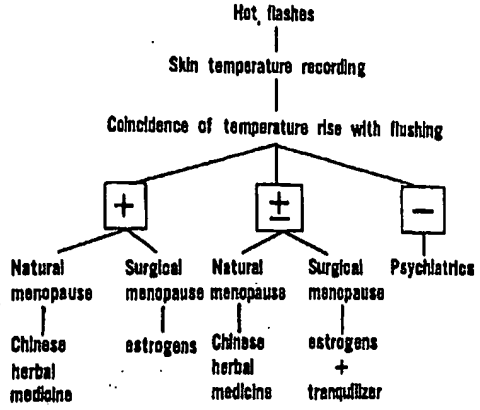


Fig. 5. Evaluation and treatment of women with hot flashes.



because of the potential risk of carcinogenesis of estrogens; on the other hand, estrogens can be prescribed liberally if hot flashes result from surgical menopause.

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Technologie pflanzlicher Arzneizubereitungen

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und Prof. Dr. Peter C. Schmidt,
Institut für Pharmazeutische Technologie der Universität Marburg

Mit 232 Abbildungen und 67 Tabellen

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Vorwort

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Kapitel 7

Die Weiterverarbeitung zum Fertigarzneimittel

Nach § 4 AMG sind Fertigarzneimittel Arzneimittel, die im voraus hergestellt und in einer zur Abgabe an den Verbraucher bestimmten Packung in den Verkehr gebracht werden. Insofern zählen pflanzliche Arzneizubereitungen wie abgepackte Teemischungen, tassenfertige Wirkstoffextrakte sowie manche Fluidextrakte oder Tinkturen zu den Fertigarzneimitteln. In den meisten Fällen aber handelt es sich um Zwischenprodukte oder Halbzeug, die zu flüssigen oder festen Arzneiformen weiterverarbeitet werden müssen. Die dabei auftretenden Schwierigkeiten sind häufig größer als bei der Verarbeitung von Einzelstoffen natürlichen oder synthetischen Ursprungs.

7.1 Herstellung fester Arzneiformen aus Trockenextrakten

Die Verarbeitung von Pflanzenextrakten zu Weichgelatinekapseln verursacht im allgemeinen keine besonderen Schwierigkeiten. Die meist hydrophilen Extrakte lassen sich fein gepulvert leicht mit lipophilen Dispersionsmitteln in pumpbare Suspensionen überführen und nach dem Scherer-Verfahren verkapseln.

Dagegen stehen einer Direktabfüllung in Hartgelatinekapseln die schlechten Fließeigenschaften der meisten Trockenextrakte entgegen. Lyophilisierte und sprühgetrocknete Extrakte sind darüber hinaus viel zu voluminös, d. h. ihre Schüttdichte ist zu gering, um jeweils eine Einzeldosis in Kapseln der üblichen Größen abfüllen zu können. Es muß also wie bei der Herstellung von Tabletten (s. u.) granuliert werden.

Zu den vorstehend erwähnten schlechten Fließeigenschaften und der geringen Schüttdichte kommen noch die meist ausgeprägte Hygroskopizität und der häufig sehr tief liegende eutektische Punkt der als Vielkomponentensysteme vorliegenden Extrakte. Gerade diese Eigenschaften erschweren die Tablettierung und machen eine Direktverpressung meistens unmöglich.

Schon sehr früh bemühte sich die Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik (APV), brauchbare Vorschriften zu erarbeiten. In Tab. 7.1 sind die in einem Fortbildungslehrgang 1961 (s. 7.4.1) verwendeten Rezepturen wiedergegeben. Sie sind gekennzeichnet durch den Zusatz von hochdisperser Kieselsäure, Aerosil®, das die Hygroskopizität der Extrakte mindert und das Pulver auflockert. Auch die Verwendung von hydrophobiertem Aerosil® R 972 wurde beschrieben. Befriedigende Tabletten wurden nach folgender Vorschrift erzielt (7.4.2):

Trockenextrakt (+ 5% Aerosil® R 972)	45 %
Kartoffelstärke	10 %
Aerosil® R 972	2 %
Talcum siliconisatum	3 %

Das hydrophobierte Aerosil® R 972 wird mit dem Extrakt gemischt. Es soll den hygroskopischen Extrakt vor dem Eindringen von Wasser und damit vor dem

Tab. 7.1: Rezepturen für die Extrakt-Tabletten nach 7.4.1

Modellvorschrift:		
A	Pflanzenextrakt (Extract veget.)	150,0
	Aerosil purum	20,0
	Amylum solani	20,0
a	gut vermischen und sieben	
b	Durchfeuchten mit Glycerinalkohol 2% ig	
c	Granulation mit Cetyl-Isopropanol 10% ig ca. 20,0 =	2,0 Tr. S.
d	Dispergierung	
e	Trocknung	
B	Äußere Phase Gleitmittel	8,0
		200,0
	1000 Kerne à 0,2	
Vorschriften:		
AESCULUS COMP. 0,2		
A I	Aneurin Vitamin B1	2,0
	Extract. Aesculi hippocast.	123,0
	Extract. Hamamelis	25,0
	Aerosil pur.	20,0
	Amylum solani	18,0
a	durchfeuchten mit Glycerin Isopropanol und abtrocknen	
b	Feuchtgranulation Cetyl Isopropanol 10% ca. 20,0	2,0 Tr. S.
c	Dispergierung und Trocknung	
B	Äußere Phase Gleitmittel	10,0
		200,0
	1000 Kerne à 0,2	
CARDIOTONICUM 0,26		
A I	Rutin	20,0
	Extract. Crategii 1 + 1	90,0
	Extract. Visci	35,0
	Extract. Allii	35,0
	Amyl. solani	38,0
	Aerosil purum	30,0
a	in einem Mixgerät gut mischen und sieben	
b	durchfluten mit Glycerin Isopropanol 2% ig q. s.	
c	Trocknen	
d	Granulation mit Cetylisopropanol 10% ig ca. 20,0	2,0 Tr. S.
e	Dispergierung und Trocknung	
B	Äußere Phase Gleitmittel	10,0
		260,0
	1000 Kerne à 0,26	

Tab. 7.1: (Fortsetzun

SEDATIVUM	
A I	Extract. Va
	Aerosil pu
	Amylum s
a	Durchfeuc
b	Granulatio
c	Dispergier
B	Äußere Pl

HEPATICUM-C	
A I	Extract, a
	Fel. tauri
	Rhiz. cur
	Extract.
	Extract.
	Extract.
	Extract.
	Aerosil
	Amylum
a	im Mixg
b	durchflu
c	Feuchtg
	Cetyliso
d	Disperg
B	Äußere

LAXATIVUM	
A I	Extrac
	Extrac
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	Aeros
a	Durch
b	Gran
c	Dispe
B	Äuße

Tab. 7.1: (Fortsetzung)

SEDATIVUM			
A	I	Extract. Valerianae	150,0
		Aerosil pur.	20,0
		Amylum solani	20,0
	a	Durchfeuchten mit Glycerin Isopropanol und abtrocknen	
	b	Granulation mit Cetyl Isopropanol 10%	
		20,0	2,0
	c	Dispergierung und Trocknung	
B		Äußere Phase Gleitmittel	8,0
			200,0
		1000 Kerne à 0,2	
HEPATICUM-CHOLAGOGUM 0,29			
A	I	Extract, aloes	60,0
		Fel. tauri	20,0
		Rhiz. curcum.	50,0
		Extract. gentian.	15,0
		Extract. chelidonii	10,0
		Extract. chinae	10,0
		Extract. cynarae	50,0
		Aerosil	35,0
		Amylum solani	27,0
	a	im Mixgerät gut vermischen und sieben	
	b	durchfluten mit Glycerin Isopropanol qu. s.	
	c	Feuchtgranulation	
		Cetylisopropanol ca. 30,0	3,0 Tr. S.
	d	Dispergierung und Trocknung	
B		Äußere Phase Gleitmittel	10,0
			290,0
		1000 Kerne à 0,29	
LAXATIVUM COMP. 0,21			
A	I	Extract. aloes	60,0
		Extract. rhei	60,0
		Extract. cascar. sagrad.	25,0
		Diacetyldioxyplenylisatin	5,0
		Fel. tauri sicc.	15,0
		Aerosil pur.	24,0
	a	Durchfeuchten mit Glycerin Isopropanol und abtrocknen	
	b	Granulation mit Cetyl Isopropanol 10%	
		ca. 10,0	1,0 Tr. S.
	c	Dispergierung und Trocknung	
B		Äußere Phase Gleitmittel	10,0
			210,0
		1000 Kerne à 0,21	

Verkleben schützen. Diese Vormischung wird mit den anderen Bestandteilen vermischt und brikettiert. Die Briketts werden zerkleinert und folgende weitere Bestandteile hinzugemischt:

Granulatum simplex	35 %
Talcum siliconisatum	3 %
Aerosil compositum	2 %

Die resultierenden Tabletten haben bei einer Bruchfestigkeit von 2 $kg \cdot cm^{-2}$ eine Friabilität von 0,75 % bei einer Zerfallszeit von weniger als 6 Minuten.

CRIPPA (7.4.3) hat Formulierungsfaktoren am Beispiel eines Cascara-Trockenextraktes eingehend untersucht. Er konnte zeigen, daß die Verwendung von Wasser an Stelle von Chloroform als Granulierflüssigkeit mit Polyvinylpyrrolidon als Bindemittel bei sonst unveränderter Rezeptur zu einer starken Erhöhung der Zerfallszeit führt.

Zur Erzielung einwandfreier Tabletten wird außerdem der Zusatz von kolloidem Siliciumdioxid und Methylencasein vorgeschlagen, die eine Senkung der Zerfallszeit bewirken.

Einen Rezepturvorschlag gibt Tab. 7.2 wieder:

Tab. 7.2: Tablettenrezeptur für Cascarra-Extrakt

Zusammensetzung	Mengen/Tabl.
Cascara-Trockenextrakt, granuliert	125 mg
Lactose	60 mg
Magnesiumstearat	1,5 mg
kolloidales Siliciumdioxid	5 mg
Mikrokristalline Cellulose	8,5 mg
Tablettengewicht	200,0 mg
Härte	30–35 N
Friabilität	0,25 %
Zerfall	10–15 min

Tabelle 7.3: Baldrianextrakt-Tabletten

Zusammensetzung	Rezeptur Nr. 1 (Mengen in %)	Rezeptur Nr. 2 (Mengen in %)
Baldrianextrakt, gefriergetrocknet	20,0	40,0
kolloides Siliciumdioxid***	1,0	1,0
mikrofeine Cellulose, granuliert*	59,0	40,0
mikrofeine Cellulose, gepulvert**	20,0	9,4
Reisstärke	-	9,6
Tablettengewicht	250 mg	122 mg
Zerfallszeit	18 min	4 min 48 s
Bruchfestigkeit (Heberlein Tester)	170 N	58 N
Frialilität (Roche-Friabilator)	0,05 %	0,05 %

* Elcema G 250, ** Elcema P 100, *** Aerosil 200

CRIPPA (7.4.3) ziel
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CRIPPA (7.4.3) zielt bei seinen Vorschlägen darauf ab, durch den Zusatz von Adsorbentien wie hochdisperser Kieselsäure Wasser zu binden und dem Preßling eine gewisse Porosität zu verleihen. Dieser Effekt wird durch die Verwendung von mikrofeiner Cellulose als Füll- und Sprengmittel noch verstärkt, wie GRAF und BORNKESSEL (7.4.4) am Beispiel von Tabletten mit einem gefriergetrockneten Baldrianextrakt zeigen konnten. Tabelle 7.3 zeigt ihre Ergebnisse.

Das veränderte Mengenverhältnis Baldrianextrakt/Elcema führt in Verbindung mit dem niedrigeren Preßdruck (geringere Bruchfestigkeit) zu Tabletten mit für extrakthaltige Produkte sehr guten Zerfallszeiten und Friabilitäten, die auch bei Lagerung erhalten bleiben.

Der positive Einfluß von mikrofeiner und mikrokristalliner Cellulose konnte in eigenen Versuchen (7.4.5) mit extrakthaltigen Tabletten bestätigt werden. Dabei wurde außerdem festgestellt, daß der ursprünglich von KEYMER (7.4.2) vorgeschlagenen Hydrophobierung des Extraktes vor der Auflockerung mit kolloider Kieselsäure der Vorzug zu geben ist. Ein mit Magnesiumstearatzusatz vermahlener Extrakt zeigt gegenüber einem mit kolloidem Siliciumdioxid behandelten eine deutlich geringere Hygroskopizität. Offensichtlich führt die Vermahlung auf einer hochtourigen Stiftmühle in Gegenwert von ca. 10–15 % Magnesiumstearat zu einer deutlichen Herabsetzung der Benetzbarkeit.

Das anfallende Produkt ist ein lockeres, fließendes Pulver. Diese Maßnahmen in Kombination mit der Verwendung von mikrokristalliner oder mikrofeiner Cellulose können für eine Vielzahl von Extrakten zur Tablettierung empfohlen werden.

Die Verwendung von Magnesiumstearat wirft natürlich die Frage nach der Bioverfügbarkeit auf. Bekanntlich kann durch hydrophobierende Substanzen sowohl die Zerfallszeit als auch die Auflösungsgeschwindigkeit negativ beeinflußt werden.

In diesem Zusammenhang ist eine Arbeit von BURGER und DIALER (7.4.6) interessant. Die Autoren untersuchten eine Reihe von Baldrianwurzeln enthaltenden Handelspräparaten auf ihre Zerfallszeit und Auflösungsgeschwindigkeit.

Die Präparatereihe wurde ausgewählt, weil man bei einem Schlaf- und Beruhigungsmittel einen schnellen Wirkungseintritt wünscht und deshalb an kurzen Zerfallszeiten und schneller Wirkstofffreisetzung interessiert ist.

Alle Präparate waren Zuckerdragees. Die Aufdragierung betrug zwischen ca. 400 und 800 % bezogen auf das Kerngewicht, was offensichtlich zur Geruchsabdeckung notwendig ist. Von allen acht untersuchten Handelspräparaten erfüllte nur eines die Anforderungen der Arzneibücher an die Zerfallszeit. Aufgrund der ermittelten Auflösungsgeschwindigkeiten kommen die Autoren zu dem Schluß, daß für Trockenextrakte enthaltende feste Arzneizubereitungen in künftigen Arzneibüchern eigene Monographien geschaffen werden sollten. Für die Bioverfügbarkeit solcher Zubereitungen sei ausschließlich die Zerfallszeit relevant.

D12

ASHP Statement on Unit Dose Drug Distribution

The unit dose system of medication distribution is a pharmacy-coordinated method of dispensing and controlling medications in organized health-care settings.

The unit dose system may differ in form, depending on the specific needs of the organization. However, the following distinctive elements are basic to all unit dose systems: medications are contained in single unit packages; they are dispensed in as ready-to-administer form as possible; and for most medications, not more than a 24-hour supply[a] of doses is delivered to or available at the patient-care area at any time.[1,2]

Numerous studies concerning unit dose drug distribution systems have been published over the past several decades. These studies indicate categorically that unit dose systems, with respect to other drug distribution methods, are (1) safer for the patient, (2) more efficient and economical for the organization, and (3) a more effective method of utilizing professional resources.

More specifically, the inherent advantages of unit dose systems over alternative distribution procedures are

1. A reduction in the incidence of medication errors.
2. A decrease in the total cost of medication-related activities.
3. A more efficient usage of pharmacy and nursing personnel, allowing for more direct patient-care involvement by pharmacists and nurses.
4. Improved overall drug control and drug use monitoring.
5. More accurate patient billings for drugs.
6. The elimination or minimization of drug credits.
7. Greater control by the pharmacist over pharmacy workload patterns and staff scheduling.
8. A reduction in the size of drug inventories located in patient-care areas.
9. Greater adaptability to computerized and automated procedures.

In view of these demonstrated benefits, the American Society of Hospital Pharmacists considers the unit dose system to be an essential part of drug distribution and control in organized health-care settings in which drug therapy is an integral component of health-care delivery.

References

1. Summerfield MR. Unit dose primer. Bethesda, MD: American Society of Hospital Pharmacists; 1983.
2. American Society of Hospital Pharmacists. ASHP technical assistance bulletin on hospital drug distribution and control. *Am J Hosp Pharm.* 1980; 37:1097–1103.

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[a]In long-term care facilities, a larger supply of medication (e.g., 48 or 72 hours) may be acceptable.

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D13

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Ministerium für Hoch- und Fachschulwesen

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Voigt, R.
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Berlin
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Vorwort zur 5. Auflage

Die freundliche Aufnahme, die die Erstauflage des Lehrbuchs der pharmazeutischen Technologie (1973) im In- und Ausland fand, führte innerhalb des vergangenen Jahrzehnts, bedingt durch Lizenz- und Übersetzungsauflagen, zu insgesamt 10 Ausgaben. Hierbei erfuhr das Buch eine kontinuierliche Überarbeitung und eine ständige Anpassung an die jeweilige Arzneibuchsituation des Vertriebsraums.

Die vorliegende 5. Auflage ist völlig überarbeitet und vielfach erweitert worden. Von den vorgenommenen Veränderungen sind mehr oder weniger alle Kapitel betroffen. Ergänzt wurden vor allem die Kapitel Statistische Versuchsplanung, Trocknen, Granulierung, Augenarzneien, Emulsionen, Suspensionen, Aerosole, Pflanzenextraktion. Neugefaßt sind die Kapitel Kapseln, Entkeimungsfiltration, Aseptisches Arbeiten sowie Löslichkeit, Lösungsgeschwindigkeit, Lösungsverbesserung. Neu gestaltet, aktualisiert und erweitert ist der Buchteil Biopharmazie einschließlich Therapeutische Systeme. Ein Kapitel Potentielle Arzneiformen wurde angefügt.

Eine Anzahl Tabellen entfielen, eine etwa gleich große Zahl wurde neu aufgenommen.

Ein Austausch ergab sich auch bei den Abbildungen, darüber hinaus aber erfolgten Neuaufnahmen, so daß sich die Zahl der Abbildungen von 264 auf 279 erhöhte.

Der Hauptteil des Buches, der die Prinzipien und den gegenwärtigen Stand der pharmazeutischen Technologie wiedergibt, ist frei von Bezügen zu nationalen und damit nur regional verbindlichen gesetzlichen Festlegungen. Da andererseits das jeweils gültige Arzneibuch in die pharmazeutisch-technologische Hochschulausbildung und Weiterbildung sowie in die tägliche Arbeit von pharmazeutischen Technologen in Forschung und Arzneimittelproduktion voll einbezogen ist, sind konkrete Angaben hierüber unerläßlich. Die im Anhang aufgeführten und den Kapiteln des Hauptteils zugeordneten pharmazeutisch-technologisch relevanten Arzneibuchauszüge sollen dem Leser den Zugriff zu diesen Informationen erleichtern. Die im Anhang zusammengestellten Definitionen, Angaben, Prüfungen und Präparate beziehen sich nunmehr auf die neueste Ausgabe des Arzneibuchs der Deutschen Demokratischen Republik (AB-DDR 83). Die hier fixierten Festlegungen entsprechen zugleich weitgehend dem Compendium Medicamentorum des Rates für gegenseitige Wirtschaftshilfe (CM-RGW).

Die neue Auflage ist hinsichtlich der Schreibweise chemischer Begriffe umgestellt worden, wobei die Empfehlungen der IUPAC zur chemischen Nomenklatur weitgehend Berücksichtigung fanden.

Neben den SI-Einheiten (Système International d'Unités) sind auch in dieser Auflage die alten Einheiten weiterhin angeführt, da in der gegenwärtigen Übergangsphase zwangsläufig Meßmittel mit Skalenteilung in bisherigen Einheiten in Gebrauch sind, zudem ist eine konsequente Anwendung der neuen Einheiten in den Arzneibüchern nur langfristig möglich.

Allen Fachkollegen, die mir Hinweise und Anregungen für die neue Auflage zukommen ließen und die weitgehend Berücksichtigung fanden, danke ich verbindlich.

Wie bei den vorangegangenen Auflagen erhielt ich auch diesmal bewährte Unterstützung. Zu danken habe ich Herrn Dr. sc. nat. M. BORNSCHEIN für Diskussionen und engagierte Mitarbeit und Herrn Chem.-Ing. H. DÖHNERT für die zeichnerische Gestaltung der neu aufgenommenen Abbildungen. Dem Verlag und insbesondere der Lektorin Frau Dipl.-Biol. LIESELOTTE WIETSTRUCK gilt mein Dank für alle Bemühungen und für eine erfreuliche Zusammenarbeit.

Berlin R. VOIGT

Vorwort zur 1. Auflage (gekürzt)

Unter den Disziplinen der Arzneimittelwissenschaften hat sich die pharmazeutische Technologie in den letzten Jahrzehnten am stärksten entwickelt. Sie ging aus der galenischen Pharmazie (Galenik) hervor, worunter man die überwiegend in der Apotheke vorgenommene Arzneianfertigung nach ärztlichen Rezepten und nach Vorschriften der Arzneibücher verstand. Ureigenstes Anliegen der Pharmazie war von alters her, Arzneistoffe zu geeigneten, gebrauchsfertigen Arzneizubereitungen (Arzneiformen) zu verarbeiten und damit Applikationsformen zu schaffen, die am Patienten zur Anwendung kommen. In immer stärkerem Maße verlagerte sich in neuerer Zeit die Herstellung von Arzneiformen in den halbindustriellen und industriellen Bereich. Damit löste sich das Fach endgültig von der oft noch auf Empirie beruhenden Herstellung von Arzneizubereitungen, deren Palette sich wesentlich durch moderne Arzneiformen erweiterte. Neue Wirkstoffträger und Hilfsstoffe, neu entwickelte Arbeitsverfahren und der hiermit verbundene ständige Fortschritt auf dem Sektor der Apparate-, Maschinen- und Automatentechnik sowie die kontinuierliche Verbesserung der Methoden und Geräte zur Prüfung von Arzneiformen gaben diesem pharmazeutischen Fachgebiet, für das im internationalen Rahmen auch weitere Bezeichnungen, wie Arzneiformentechnologie, Arzneiformung, pharmazeutische Technik, pharmazeutische Verfahrenstechnik u. a. üblich sind, ein neues Profil. Bei der Schaffung optimaler Arzneiformen sind darüber hinaus in jedem Falle auch biopharmazeutische Aspekte in der pharmazeutischen Technologie zu berücksichtigen, da die Wirkungsintensität und -dauer eines Arzneistoffs im wesentlichen Maße durch die Arzneiform, und zwar sowohl durch die eingesetzten Grund- und Hilfsstoffe als auch durch die angewendete Verfahrenstechnik beeinflußbar sind.

Das vorliegende Buch beinhaltet alle Teilgebiete der pharmazeutischen Technologie, wobei es darauf ankam, sie in einem ausgewogenen Verhältnis zur Darstellung zu bringen. Die Stoffülle und Heterogenität des Fachgebiets erforderte eine konsequente Begrenzung des Darzubietenden und eine Konzentrierung auf das Wesentliche. So wurde bewußt auf die Erörterung einiger älterer, nicht mehr zeitgemäßer Arzneizubereitungen verzichtet. Den Umfang der Darstellung der Einzelarzneiformen bestimmte die jeweilige Bedeutung und der Entwicklungstrend. Den theore-

Gasförmige Arzneiformen

21. Aerosole/Inhalate

Durch Drogenextraktion gewonnene Arzneiformen

22. Wäßrige Auszüge, Tinkturen, Extrakte

Generelle Aspekte der Arzneiformung

22. Wäßrige Auszüge, Tinkturen, Extrakte

22.1. Allgemeines

Seit Urzeiten dienen pflanzliche und tierische Drogen bzw. hieraus durch Extraktion hergestellte Arzneizubereitungen zur Heilung von Mensch und Tier. Auch heute, wo in immer stärkerem Maße synthetisch gewonnene Arzneimittel zu Arzneiformen verarbeitet werden und die Bemühungen dahin gehen, möglichst chemisch und pharmakologisch eindeutig definierte Arzneimittel therapeutisch einzusetzen, bilden Drogen nach wie vor in erheblichem Umfang das Ausgangsprodukt für Arzneiformen. Allerdings entfielen ursprünglich etwa 70–80 % der Arzneibuchartikel auf pflanzliche Drogen, heute weisen die europäischen Pharmakopöen nur noch einen Anteil von 10–20 % auf. In den USA liegt der Anteil bei 5 %. Pflanzliche Wirkstoffe werden in den letzten Jahrzehnten in zunehmendem Maße zunächst in reiner Form isoliert und dann zu entsprechenden Arzneipräparaten verarbeitet. Dennoch ist die Zahl der pharmazeutisch genutzten Zubereitungen hoch, in denen die aus dem Drogenmaterial gewonnenen Wirkstoffe als Komplex vorliegen. Die Drogenkomponenten kommen in diesen Fällen in mehr oder weniger „ungereinigter" Form zur Anwendung. Auch in den neuesten Pharmakopöen ist die Zahl der pflanzlichen Zubereitungen, wenn auch eine Rückläufigkeit zu verzeichnen ist, bemerkenswert. Berücksichtigt man sowohl die galenischen Zubereitungen als auch Reinstoffe, bei denen Pflanzen das Ausgangsmaterial darstellen, so weisen die mitteleuropäischen Arzneibücher einen Anteil von rund 40 % auf.

Durch Drogenextraktion gewonnene Arzneiformen sind in der Vergangenheit z. T. durchaus berechtigt in Mißkredit gekommen. Es muß eingeräumt werden, daß nicht alle auch heute noch gebräuchlichen Zubereitungsformen als zeitgemäß einzuschätzen sind. Bei manchen ist die Haltbarkeit der Wirkstoffe nicht in ausreichendem Maße zu sichern, bei anderen liegt keine optimale Stabilität der Arzneiform selbst vor. Eine chemische Standardisierung und Normung pflanzlicher Präparate war zudem in der Vergangenheit recht kompliziert, so daß eine hinreichende qualitative und quantitative Erfassung der Wirkstoffe nicht immer möglich, zumindest aber sehr arbeitsaufwendig war. Auch die Ausgangsdrogen waren im Hinblick auf ihre qualitative und quantitative Wirkstoffzusammensetzung und auf ihre Haltbarkeit selbst in Arzneibuchmonographien nicht eindeutig fixiert.

Mittels pharmakologischer Testungen sind Reinsubstanzen erfahrungsgemäß recht gut zu beurteilen. Bei Wirkstoffkomplexen, wie sie in Drogen und den hieraus gewonnenen Arzneiformen vorliegen, sind dagegen verbindliche Aussagen meist nicht zu treffen, so daß von pharmakologischer Seite eine konkrete Bewertung dieser Arzneiformen kaum zu erwarten war. Nicht selten lehnten Pharmakologen aus den genannten Gründen durch Drogenextraktion gewonnene Arzneiformen ab und wiesen ihnen oftmals lediglich eine Plazebowirkung zu. Das geschah selbst dann, wenn es sich um offensichtlich seit Jahrhunderten in der Therapie bewährte Zubereitungen handelte. So war es z. B. mangels geeigneter pharmakologischer Methoden nicht möglich, die

sedative Wirkung des Baldrians und seiner Zubereitungen zu erfassen. Oft wurde daher diesem auch in der Volksheilkunde geschätztem Mittel von dieser Seite jeglicher therapeutische Effekt abgesprochen. Erst in neuerer Zeit gelang die Isolierung und Reindarstellung des wirksamen Prinzips des Baldrians. Hierbei handelt es sich um Verbindungen, die als Valepotriate bzw. Halazuchrome bezeichnet werden. Sie sind in der Droge in Mengen zwischen 0,5 und 2% enthalten und infolge ihrer Alkohollöslichkeit in entsprechende Arzneiformen überführbar. Die therapeutische Wirksamkeit von Arzneiformen mit ausreichendem Gehalt an diesen Inhaltsstoffen des Baldrians steht nunmehr außer Frage.

Heute unterliegen pflanzliche Arzneibuchzubereitungen wie auch die Drogen selbst einer exakten chemischen Überprüfung. Moderne analytische Verfahren, nicht zuletzt die Dünnschichtchromatographie, geben in qualitativer und quantitativer Hinsicht Gewähr für eine weitgehende Standardisierung der Präparation. Dennoch wird die Frage bestehenbleiben, ob es im Einzelfall günstiger ist, eine Arzneiform mit einer chemisch reinen Verbindung zur therapeutischen Anwendung zu bringen oder eine Arzneiform mit einem pflanzlichen Vielstoffgemisch.

Im allgemeinen kann pflanzlichen Zubereitungen nachgesagt werden, daß sie eine milde aber durchaus sichere Wirkung erbringen. Pflanzliche Laxantien werden z. B. oftmals mehr geschätzt als synthetische Abführmittel. Während bei synthetisch gewonnenen Verbindungen Nebenwirkungen in immer stärkerem Maße festzustellen sind, fehlen diese bei Arzneiformen, die aus pflanzlichen Materialien gewonnen werden. Als Positivum für die letzteren wird oftmals angeführt, daß sich die komplexe Wirkung, die durch die Vielzahl der vorliegenden Inhaltsstoffe bedingt ist, als Vorteil für die Gesamtwirkung erweist. Sicherlich wird der therapeutische Effekt ein anderer, möglicherweise auch ein günstigerer sein, wenn nicht nur ein isoliertes Arzneimittel auf den Organismus einwirkt, sondern Haupt- und Nebenstoffe gemeinsam agieren. Wesentlich ist, daß auch in der Arzneiform vorliegende Pflanzeninhaltsstoffe, die nicht als Wirkstoffe anzusprechen sind, die Arzneimittelwirkung in nicht unbeträchtlichem Maße zu beeinflussen vermögen. Während z. B. Saponine resorptionsbeschleunigend wirken, können Gerbstoffe den Eintritt der Wirkung verzögern oder diese auch verlängern.

Abgesehen von Teemischungen und von der relativ seltenen Anwendung gepulverter Drogen in Form von eingestellten Drogenpulvern (Pulveres titrati), die nach chemisch oder biologisch bestimmtem Wirkstoffgehalt mit indifferenten Stoffen auf den vorgeschriebenen Wert eingestellt sind, sowie von Tabletten und Kapseln, dienen pflanzliche Materialien im allgemeinen zur Herstellung folgender Arzneiformen, die als applizierbare Zubereitungen dienen oder Zwischenprodukte bei der Bereitung weiterer Arzneiformen darstellen: Aufgüsse, Dekokte, Mazerate, Tinkturen, Extrakte, aromatische Wässer, Arzneispiritusse, Arzneiöle, Weine, Sirupe, Auszugssalben.

Die Herstellung zeitgemäßer Arzneiformen, die Arzneistoffe aus pflanzlichem oder tierischem Material beinhalten, setzt die Einhaltung folgender Forderungen voraus:

- Sicherung der Gewinnung der Wirkstoffe in möglichst unveränderter Form aus hochwertigem und gleichförmigem Ausgangsmaterial, das den Arzneibuchforderungen entspricht,
- Erzielung hoher Ausbeutewerte,
- Gewährleistung einer langfristigen Erhaltung des Wirkstoffgehalts (Stabilität der Wirkstoffe während des Herstellungsprozesses und während der Lagerung) durch Wahl geeigneter Herstellungstechnologien und entsprechender Arzneiformen,
- Schaffung einer standardisierten Arzneiform.

22.2. Die Droge als Ausgangsmaterial für Arzneiformen

Als Ausgangsmaterialien zur Herstellung von Arzneiformen dienen Frischpflanzen im allgemeinen jedoch getrocknete Pflanzen, Pflanzenteile sowie pflanzliche Rohprodukte (Harze, Milchsäfte). Voraussetzung für die Schaffung vollwertiger Arzneiformen ist eine hohe Qualität der Drogen, die durch die Festlegungen in den Monographien der Arzneibücher fixiert ist. Häufig ist der Gehalt an Drogeninhaltsstoffen durch eine untere und eine obere Grenze festgelegt. Bei Drogen mit stark wirkenden Arzneimitteln sind diese Grenzen sehr eng gezogen, oder aber es wird ein bestimmter Wert als Mindestgehalt gefordert. Hierbei ist zu berücksichtigen, daß eine Standardisierung der Drogen, wie sie heute von allen Pharmakopöen angestrebt wird, unerläßlich für die Konstanz der Höhe und des Spektrums des Wirkstoffgemisches ist. Drogen, die infolge zu langer Lagerung nicht mehr den im Arzneibuch niedergelegten Forderungen entsprechen (Alterung), sind von der Verwendung auszuschließen.

Abbauprozesse in einer Droge gehen zumeist mit einer Minderung des Wirkstoffgehalts einher. Hierbei kann es sich um enzymatische Vorgänge, um hydrolytische Prozesse, sowie um Oxidations- und Reduktionsreaktionen handeln. Licht und Temperatur sind weitere Faktoren, die auf die Drogenhaltbarkeit einwirken. Die Qualität der Droge hängt weiterhin in ganz besonderem Maße von der Beschaffenheit der Frischpflanze ab. Wirkstoffqualität und -quantität sind nicht nur abhängig von dem z. T. genetisch fixierten Stoffwechsel, sie unterliegen auch in starkem Maße Umwelteinflüssen. Standort, Bodenbeschaffenheit, Düngung, Belichtung, Höhenlage, Klima, Witterung und Zeitpunkt der Ernte sind einige Faktoren, die den Wert einer Droge entscheidend mitbestimmen. Die Festlegung des optimalen Zeitpunkts für die Ernte bereitet dem Arzneipflanzenanbau oft erhebliche Schwierigkeiten.

Die Qualität der Droge wird darüber hinaus entscheidend beeinflußt durch die Art des Trocknungsprozesses, der sich an die Ernte anschließt. Man unterscheidet u. a. Lufttrocknung (Schattentrocknung) und Wärmetrocknung. Beim Trocknen bzw. beim Welken des Pflanzenmaterials unterliegen die Inhaltsstoffe recht unterschiedlichen Veränderungen. Enzymatische Prozesse, die die Lebensvorgänge der Pflanze sicherten, kommen auch nach der Ernte nicht sofort zum Stillstand. Durch den trocknungsbedingten Wasserverlust in der Zelle können z. B. verstärkt enzymatische Abbauprozesse bei Glycosiden ablaufen, die zur Spaltung derselben führen. Die entstehenden Aglycone sind meist wirkungslos, zumindest aber wirkungsschwächer. Der zunehmende Wasserverlust bedingt Schädigungen der Plasmastrukturen und Veränderungen der Permeabilitätsverhältnisse in der Zelle und führt dazu, daß in den verschiedenen Plasmaorganellen getrennt lokalisierte Substrate und Enzymgarnituren in Kontakt treten können, so daß enzymatische Prozesse neben- und durcheinander ablaufen („Freilauf der Enzyme"). Erfassen die Schädigungen auch die Zellwände parenchymatischer Zellen, so können Bestandteile verschiedener Zellen in Berührung kommen. Derartige nekrobiotische Prozesse enzymatischer und nichtenzymatischer Art können durch Oxidation, hydrolytische Spaltung oder Razemisierung zu unerwünschten Veränderungen arzneilich wirksamer Pflanzenstoffe führen (Phenolglycoside, Digitaloide, Senfölglycoside, Hyoscyamin, Morphin, Ascorbinsäure). Der charakteristische Geruch einiger Drogen (Waldmeister, Baldrian, Hopfen) entwickelt sich gleichfalls erst während der Trocknungsperiode bzw. der Aufbewahrung. Bei der Trocknung der Arzneipflanze tritt ein erheblicher Wasserverlust ein, doch weisen auch lagernde Drogen stets noch einen Feuchtigkeitsgehalt von einigen Prozent auf. Der Wassergehalt kann bei trockenen Drogen bis zu 10 % und mehr be-

24. Stabilität und Stabilisierung

24.1. Allgemeines

Fragen der Stabilität und Stabilisierung von Arzneistoffen und Arzneiformen haben in den letzten Jahrzehnten immer größere Bedeutung erlangt. Das hat seinen Grund in der verstärkten Einführung moderner, hochwirksamer, leider aber oft instabiler Arzneistoffe, wie Antibiotika, Fermente und Hormone, sowie in der verstärkten industriellen Produktion von Arzneifertigwaren, die zur Gewährleistung der Lagerhaltung und unter Berücksichtigung des Verteilerweges eine befriedigende Haltbarkeit aufweisen müssen. Aber auch die uns heute zur Verfügung stehenden empfindlichen Analysenverfahren haben wesentlich dazu beigetragen, Stabilitätskriterien strenger zu fassen und erhöhte Haltbarkeitsforderungen zu stellen. Zudem besitzt die Abklärung von Stabilitätsfragen nicht nur Interesse für die Bestimmung und Festlegung von Haltbarkeitsfristen, sondern ist darüber hinaus für eine exakte Auswertung biopharmazeutischer Versuche (z. B. Metabolisierung, Distributionsverhalten von Arzneistoffen) unbedingte Voraussetzung.

Stabilitätsuntersuchungen sind daher bereits bei der Entwicklung neuer Arzneimittel unerläßlich. Erste orientierende Versuche, die mit der Substanz und als Lösung durchgeführt werden, haben zum Ziel abzuklären, ob die Verbindung so stabil ist, daß weitere Entwicklungsarbeiten vertretbar sind, und um Fehlinterpretationen der Ergebnisse der pharmakologischen und technologischen Testung auszuschließen. Die zweite Stufe stellt ein screening zur Auffindung der möglichst optimalen Rezeptur dar. Hier kommt es darauf an, die Stabilität des Arzneistoffs in Anwesenheit von Hilfsstoffen und unter Berücksichtigung der Herstellungstechnologie abzuklären und gegebenenfalls geeignete Stabilisierungsmaßnahmen aufzufinden. Als rationelle Methode bietet sich die faktorielle Versuchsplanung an (s. 6.7.). Schließlich muß das formulierte Arzneimittel einer abschließenden Stabilitätsprüfung unterzogen werden.

Unter Stabilität ist zu verstehen, daß sich das Arzneimittel (Arzneistoff, Arzneizubereitung), aufbewahrt unter definierten Lagerbedingungen in seiner für die Lagerung und den Verkehr bestimmten Verpackung, in seinen wesentlichen Qualitätsmerkmalen nicht oder nur in einem zulässigen Ausmaß verändert.

Wesentliche Qualitätsmerkmale sind der Wirkstoffgehalt, der galenische Zustand, einschließlich der sensorisch wahrnehmbaren Eigenschaften, die mikrobiologische und toxikologische Beschaffenheit und die therapeutische Aktivität. Das zulässige Ausmaß der Veränderungen ist für offizinelle Arzneimittel in den Arzneibüchern festgelegt. Für Arzneifertigwaren (Spezialitäten) und nichtoffizinelle Arzneimittel gelten die in den Gütevorschriften gemachten Angaben. Den Wirkstoffgehalt betreffend ist es international üblich, einen Rückgang von 10%, d. h. auf 90% des deklarierten Gehalts, zu tolerieren, sofern durch entstehende Zersetzungsprodukte die Gesamttoxizität nicht erhöht wird.

Für industriell hergestellte Arzneifertigwaren, die lange Lagerungszeiten durchlaufen, wird ein Haltbarkeitszeitraum von 5 Jahren angestrebt. Er sollte im un-

günstigsten Falle 3 Jahre betragen. Rezepturmäßig hergestellte Arzneien, die meist sofort den Patienten erreichen, sollten eine Stabilität für mindestens einige Monate aufweisen. Für letztere Präparate wäre jedoch eine Limitierung der Aufbewahrungszeit zu begrüßen.

Die Ursachen, die die Instabilität der Arzneiformen bedingen, sind zweifacher Natur. Einmal ist es die Labilität der Arznei- und Hilfsstoffe selbst, die letztlich aus ihrem chemischen und physikalisch-chemischen Bau resultiert, zum anderen sind es die äußeren Faktoren, wie Temperatur, Feuchtigkeit, Luft und Licht, die wertmindernde Reaktionen induzieren oder beschleunigen. Besondere Bedeutung kommt der Verpackung zu, vor allem dann, wenn es sich um Kunststoffbehältnisse handelt (s. 26.3.5.). Das Ausmaß, in dem die genannten Faktoren wirksam werden, ist in hohem Maße vom galenischen Typ der Zubereitung abhängig. In festen Arzneien, wie Pulvern, Pudern und Tabletten, verlaufen haltbarkeitsbeschränkende Reaktionen oft so langsam, daß sie in dem interessierenden Zeitraum keine oder nur untergeordnete Stabilitätsprobleme aufwerfen. Hingegen sind flüssige, wäßrige Präparationen, wie Injektions- und Infusionslösungen, Augen- und Nasenarzneien, Mixturen, Suspensionen und Emulsionen, aber auch wasserhaltige Systeme unterschiedlicher Konsistenz, wie Salben, Pillen, und Extrakte, für Zersetzungen prädestiniert.

Aus didaktischen Gründen wird zwischen physikalischen, chemischen und mikrobiellen Veränderungen unterschieden. Praktisch ist die exakte Zuordnung einer Instabilität zu einer dieser Kategorien oft nicht möglich, da es sich meist um ein komplexes Geschehen handelt, dessen Ergebnis erfaßbar oder wahrnehmbar wird. So ist z. B. die Verfärbung einer Epinephrinlösung ihrem Erscheinungsbild nach eine physikalische Veränderung, die aber ihre Ursache in der Bildung gefärbter Zersetzungsprodukte hat, und die daher den chemischen Veränderungen zuzuordnen ist.

24.2. Methoden zur Stabilitätsbestimmung

Unabhängig vom Charakter der ablaufenden Zersetzungsprozesse (chemische, physikalische, mikrobiologische Veränderungen) ist es wichtig zu wissen, für welche Zeit der Arzneistoff bzw. das Arzneistoffsystem unter bestimmten Umweltbedingungen die angeführten Forderungen erfüllt. Zur Erfassung der Stabilitätsverhältnisse sind zwei Methoden gebräuchlich.

24.2.1. Langzeit-Haltbarkeitstest

Bei diesem „klassischen" Test geht man so vor, daß das Arzneimittel während des interessierenden Zeitraums unter den geforderten bzw. angestrebten Lagerbedingungen (Temperatur, Licht, Luft, Feuchtigkeit) in einem Klimaschrank oder Klimaraum aufbewahrt wird. In geeigneten Zeitabständen und am Versuchsende werden der Arzneistoffgehalt bzw. der Wirkwert, die mikrobiologische Beschaffenheit sowie der sensorisch und mit physikalischen Methoden erfaßbare galenische Zustand kontrolliert. Das Verfahren ist langwierig – in der Regel 5 Jahre – und läßt im allgemeinen keine Schlüsse auf den Zersetzungsmodus zu. Unter Verwendung der nach einjähriger Lagerung erhaltenen Versuchsergebnisse kann durch Hochrechnung eine Haltbarkeitsprognose für 5 Jahre getroffen werden.

D14

V/1 Occurrence of Anabolic Agents in Plants and their Importance

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Summary

More than 40 plant species have been shown to contain substances that are active in biological assays for estrogenic activity. Such substances may be constitutive metabolic products of a plant, or be formed adaptively in response to environmental factors, such as fungal attack (e.g. coumestrol synthesis in alfalfa infected with *Pseudopeziza medicagensis*); in other instances estrogens may arise from microbial attack on plant material during storage (e.g. zearalenone formation from corn by *Fusarium* spp.). Phyto-estrogens may reach man through direct consumption of fresh fruit, vegetables and processed plant products (e.g. administration of olive or cornoil can induce vaginal keratinization in post-menopausal women); or – more relevant to this Symposium – by consumption of carcasses and products from animals fed estrogen-containing forage.

Important pasture and forage plants shown to contain phyto-oestrogens include *Trifolium subterraneum L.*, notably the cultivars Dwalganup, Mt. Barker, Yarloop and Marrar, *T. pratense* (red clover), *T. fragiferum L.* (strawberry clover), *T. alexandrinum* (berseem clover), *Medicago sativa* (alfalfa or lucerne) and *Soya hispida* (soya beans). A beneficial anabolic action of the estrogens contained in these plants has been implied, but not unequivically established. More attention has been paid to their noxious effects on livestock. On affected *T. subterraneum* pasture, castrated male sheep showed lactation, squamous metaplasia of the bulbo-urethral glands and urethral stenosis; infertility, variously attributed to suppression of gonadotrophin release and ovulation; faulty ovum transport; premature regression of corpora lutea; irreversible cystic hyperplasia of endometrial glands on prolonged exposure; dystocia and prolapse of the uterus. Sporadic incidence of phyto-estrogen induced infertility in cattle has been reported, attended by ovarian cyst formation. Estrogenic activity in forage plants has been reported from Australia, New Zealand, India, Sweden, Great Britain, Germany, Denmark, Holland, Finland, Egypt and Israel.

The clover constituents chiefly incriminated for these effects are glycosides of the isoflavone derivatives genistein and its 4'-methyl ether biochanin-A, daidzein and its 4'-methyl ether formononetin, and pratensein; coumestrol and its 3'- and 4'-methyl ethers account for the estrogenic activity of alfalfa. The isoflavone content of subterranean clover may reach 3 percent of its dry weight, and the coumestrol content of lucerne may exceed 100 µg/g. Coumestrol and genistein compete with 17β-estradiol for binding sites on the uterine cytoplasmic receptor and induce macromolecular synthesis in the uterus, but fail to induce ovum implantation in ovariectomized, gestagen-maintained rats. Uterotrophic activity of coumestrol and genistein given parenterally to sheep is approximately 10^{-3} and 10^{-5} times that of stilboestrol, respectively. Biological activity of ingested phytoestrogens is modified by ruminal micro-organisms and hepatic metabolism. The pro-estrogens 4'methylcoumestrol, biochanin-A and formononetin undergo *O*-demethylation in the rumen and liver to give rise to coumestrol, genistein and daidzein, respectively. Daidzein is further metabolized in the rumen to equol (about 70 percent) and *O*-desmethylangolensin (5–20 percent), both of which possess weak but significant estrogenic activity; genistein and biochanin-A are transformed chiefly to hormonally inert *p*-ethylphenol. The greater part of the circulating phyto-estrogens occur as glucuronide conjugates. Limited data indicate that estrogenic isoflavones and coumestans accumulate in fat depots in sheep grazing affected pasture, but the amounts reported (about 1 p.p.m.) seem too low to present a significant

health hazard to the human consumer. However, no information on the pharmacology of these substances in primates is available.

In addition to biological screening techniques, specific methods have been developed for chromatographic separation of phyto-estrogens and their determination by spectrophotometry and fluorometry, receptor radioassay or radioimmunoassay. Control measures under investigation include pasture management, selection of isoflavone-deficient mutants and active vaccination with synthetic isoflavone derivatives coupled covalently to polypeptide carriers.

1. Introduction: ecological considerations

Close to 50 plant species have by now been shown to contain substances that are active in biological assays for estrogenic activity conducted in laboratory rodents or in ruminants (for reviews see refs. 10, 15, 46, 79). Such substances may be constitutive metabolic products of the plant, or be formed adaptively in response to environmental factors, such as fungal attack. Thus coumestrol synthesis is induced or greatly augmented in alfalfa (*Medicago sativa*) on infection with the leaf-spotting organism *Pseudopeziza medicagensis*[12,70]. In other instances estrogens may arise from microbial attack during faulty storage, e.g. zearalenone is formed in corn contaminated with *Fusarium spp.*[19]. The ecological factors that determine the balance between estrogenic and non-estrogenic species in natural pastures and the remarkable seasonal and geographical variation in the estrogen content of a given clover strain have not been fully identified[10]. They include among others, the level of phosphate fertilizer applied[3,63].

Oestrogenic activity may occur in bulbs (e.g. *Allium sativum L.*[30] or tubers (e.g. *Butea superba Roxb.*[64], or be localized in the leafy parts of the plant, in its fruit or in its seed[15]. Such phyto-estrogens may reach man through direct consumption of fresh fruit, such as apples[63] and cherries[27,63], vegetables (e.g. potatoes[15]) or condiments such as garlic[30]; from hops used for beer production[90] and other processed plant products; or by consumption of carcasses and products from animals fed estrogen-containing forage. There is no published evidence that herbal estrogens reaching the human from any of these sources is of pathogenic significance. It is of interest, however, that administration of corn oil or olive oil, at the rate of 100 g per day over 10 days, was shown to cause extensive keratinization of the vaginal epithelium in post-menopausal women[84], indicating that herbal estrogens contained in these products are indeed biologically active in man and effectively absorbed from the gut.

2. Distribution and chemical nature

Important pasture and forage plants shown to contain phyto-oestrogens include *Trifolium subterraneum L.*, notably the cultivars of Dwalganup, Mt. Barker, Yarloop, Clare, Geraldton, Dinninup, Woogenellup and Marrar [8,9,13,14,25,42,43], *T. pratense* (red clover[40,87,88]), *T. fragiferum L.* (strawberry clover[61]), *T. alexandrium* (berseem clover[35,68,69]), *T. repens* (Ladino clover[55]), M. sativa (alfalfa or lucerne[11,70] and *Soya hispida* (soya beans[20,69,86]). Only in very few cases were plant estrogens found to be identical with one of the estrogenic hormones of mammals. Examples are the isolation of estrone from palm kernels by Butenandt[18], and the reported occurrence of estriol in willow catkins[78]. With these esoteric exceptions, however, the phytoestrogens thus far identified proved to be phenols not chemically related to the hormonal steroids, but they share with 17β-estradiol certain structural features (Fig. 1) that may account for their biological activity.

The clover constituents chiefly associated with estrogenic activity are glycosides[8] of the isoflavone derivatives genistein[14] and its 4'-methyl ether biochanin-A[58], daidzein and its 4'-methyl ether formononetin (Fig. 2; ref.[15,16,41,43,44,72]) and pratensein[88]; coumestrol

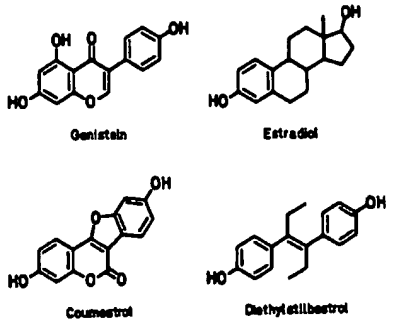

Fig. 1. Structural similarity between non-steroidal estrogens and estradiol

and its 3'- and 4'-methyl ethers account for the estrogenic activity of alfalfa and medics[10, 11, 16, 70, 75]. The isoflavon content of subterranean clover may reach 3% of its dry weight[41] and the coumestrol content of lucerne may exceed 100 $\mu g/g$[10, 69]. The uterotrophic activity of coumestrol and genistein given parenterally to sheep is approximately 10^{-3} and 10^{-5} times that of stilbestrol respectively[16]. Both compounds were also active by the intraruminal route, but potency was 1/20 (genistein) to 1/100 (coumestrol) of that observed on intramuscular administration of the same compound[16, 41].

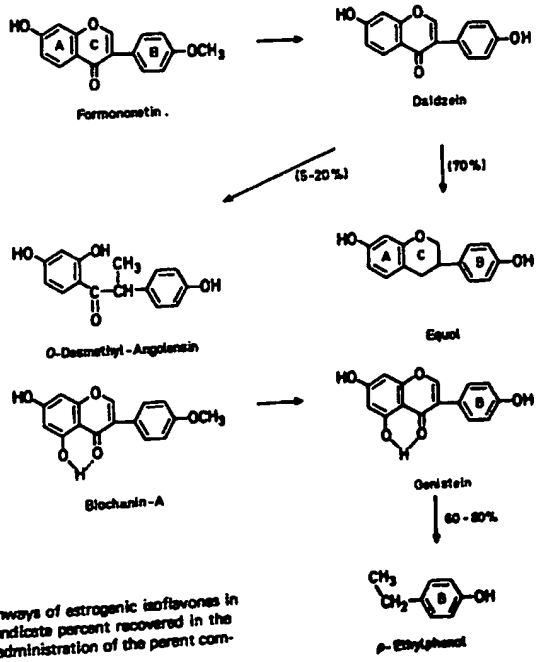

Fig. 2. Major metabolic pathways of estrogenic isoflavones in sheep. Numbers in brackets indicate percent recovered in the urine following intraruminal administration of the parent compound (ref. [73])

A recent analysis of five samples of soybean oil cake (Shemesh, Ayalon and Lindner, unpublished observations), an important ingredient of dairy-cattle diets, showed the presence (p.p.m. in dry matter ± S.E.M.) of daidzein (30.0 ± 4.7), formononetin (4.3 ± 0.2), genistein (18.6 ± 2.7), coumestrol (16.5 ± 2.9) and 4'-methylcoumestrol (0.3 ± 0.02). Berseem clover (*T. alexandrium*), which plays an important part as a cattle feed in the Middle East, was shown by bioassay to contain estrogenic activity[35,68]. Chemical analysis proved that it contains the isoflavones genistein, biochanin-A and formononetin, as well as the coumestan derivative coumestrol (Shemesh, Ayalon and Lindner, unpublished observation); the plasma of heifers fed this forage contained, in addition, daidzein, probably an *O*-demethylation product of formononetin (v.i.).

3. Mode of action

The primary interaction of estradiol with its target cells appears to involve the binding of the hormone to a cytoplasmic protein receptor, characterized by a sedimentation constant of 8S in low-salt sucrose gradients. Both coumestrol and genistein compete with 17β-estradiol for binding sites on this receptor in uterine cytosol preparations from rabbit[71] or sheep[76], but the affinity of the phyto-estrogens is considerably below that of estradiol. The affinity of their methylated derivatives (e.g. formononetin and 4'-methylcoumestrol) for the receptor is still lower, though these compounds are estrogenically active *in vivo*. Isoflavan-7,4'-diol (equol) almost equals genistein in affinity for the receptor. The significance of this compound will be discussed later in relation to the metabolism of formononetin in ruminants.

Coumestrol and genistein also simulate estradiol in stimulating macromolecular synthesis in the uterus[51]. In particular, both phyto-estrogens stimulate the *de novo* synthesis of a specific "estrogen-induced protein", demonstrable within 1 h in the cytoplasm of the rat uterus by double-labelling techniques and gel-electrophoresis (A.M. Kaye, D. Sheratzky-Sömjen and H.R. Lindner, unpublished observations). This protein[52] is considered crucial for the action of estradiol[5]. While both coumestrol and genistein exert uterotrophic activity in the rat, neither compound is able to induce ovum implantation in ovariectomized gestagen-maintained rats, suggesting that the latter response involves a different receptor mechanism[57].

4. Metabolism in grazing animals: role as pro-estrogens

The biological activity of the ingested isoflavones and coumestans is modified by ruminal microorganisms and hepatic metabolism. 4'-Methylcoumestrol, biochanin-A and formononetin undergo *O*-demethylation in the rumen and liver to give rise to coumestrol, genistein and daidzein, respectively (Fig. 2, p. 159; ref.[41,50]). Daidzein is further metabolized in the rumen to equol (about 70%) and *O*-desmethylangolensin (5–20%), both of which – notably equol – possess weak but significant estrogenic activity; genistein and biochanin-A are transformed chiefly to hormonally inert *p*-ethylphenol (Fig. 2; ref.[16,72,73,74]). Formononetin is also reduced without prior demethylation to 4'-*O*-methyl-equol, which appears in the urine following administration of formononetin to sheep (Cox, Braden and Lightfoot, personal communication). The different metabolic patterns of the 5-hydroxy and 5-deoxy isoflavones probably account for the observation that the estrogenic effects of clover pastures on grazing sheep are more closely correlated with their formononetin than with their genistein content[43,44], in spite of the greater estrogenicity of genistein in parenteral assays in laboratory rodents and sheep[16]. Formononetin and the various methylated derivatives of genistein and coumestrol are thus properly classified as pro-estrogens.

The greater part of the circulating phyto-estrogens in sheep occur as water soluble glucuronide or sulphate conjugates[73].

5. Anabolic action

A beneficial anabolic action of plant estrogens on grazing animals has been implied, but not unequivocally estabilshed. The evidence for this view is, in the main, indirect: castrate lambs grew faster when fed estrogen-containing alfalfa, or when given alfalfa extracts containing coumestrol, than those raised on a non-estrogenic diet[56]. Intact female lambs did not show this response. Again, the growth response to stilboestrol is diminished or abolished in animals consuming estrogenic pasture. This was interpreted to indicate that such animals already receive maximal estrogen-mediated anabolic stimulation from their plant diet[33]. These reports are suggestive but not conclusive, if only because dietary factors other than the plant estrogens may have confounded the results. Equally inconclusive are reports attributing the so-called "spring flush" in milk yield to the estrogen content of pasture[4,5, 32,59], or the finding that the plant estrogen coumestrol enhanced the tenderness and juiciness of lambs as judged by a chewing panel[33].

6. Noxious effects on livestock

Much more attention has been paid to the noxious effects of phyto-estrogens on farm animals, and more critical work has been done in this area. This problem was first recognized in 1946 when Bennets and Underwood[9] described massive outbreaks of infertility in sheep grazing subterranean clover in Australia. Subsequently, genistein was isolated from this clover[14]. "Clover disease" is still regarded as one of the major problems of livestock production in Australia[42,45], with about 9 million sheep at risk, and in its milder form it has been recognized as a breeding problem in many other countries. Estrogenic activity in forage plants has been reported in New Zealand[48], the Phillipines[2], Japan[55], The United States of America[10], Canada[37], Chile[31], Central Africa[82], India[36], Egypt[68], Israel[1,70], The Union of Soviet Socialist Republics[77], Finland[34,81], Sweden[49], Denmark[47], Great Britain[59,60], Belgium[26], Holland[85], Germany[65,67], Italy[23] and Czechoslovakia[21,22].

The biological effects of clover estrogens responsible for fertility impairment appear to be multiple. Ewes exposed to affected subterranean clover pastures show mild to severe degrees of infertility, attributed to faulty sperm and ovum transport and interference with ovum implantation[46], premature regression of corpora lutea[53] or cystic hyperplasia of the endometrial glands, leading to irreversible sterility on prolonged exposure[9,46]. Other disturbances include maternal dystocia and prolapse of the uterus, often followed by gangrene in the field[9]. Phyto-estrogens may suppress gonadotrophin secretion[39], possibly by interfering with the positive feedback effect of endogenous estradiol on the hypothalamus (J. Goding, personal communication). Castrated male sheep grazing estrogenic *T. subterraneum* pastures showed lactation, squamous metaplasia of the male accessory glands, at times with gross enlargement of the bulbo-urethral glands and urinary retention[9,46]. The possibility that clover estrogens may cause seminal degeneration in rams has been considered on indirect evidence[46]. Sporadic incidence of phyto-estrogen-induced infertility in cattle has been reported, usually attended by ovarian cyst formation and occasionally by nymphomania[1,23,54,60,66,80, 83,89].

7. Residues in animal carcasses

Limited data[41] indicate that estrogenic isoflavones (genistein, biochanin-A, formononetin and daidzein) accumulate in fat depots in sheep grazing estrogenic pasture or given synthetic isoflavones by intra-ruminal infusion. The amounts found in the adipose tissue (about 1 p.p.m.) exceeded the concurrent plasma concentration. Nevertheless, the concentrations reported appear too low to present a serious health hazard to the human consumer, considering the low estrogenic potencies of these substances (cf. ref. 79). However, no infor-

mation on the pharmacology of the herbal estrogens in primates is available. The possibility that such estrogens may pass into milk or milk products should also be kept in mind.

8. Methods of detection

Biological assays, using the uterine weight or vaginal cornification response in ovariectomized laboratory rodents or ewes, or the teat growth response in castrated male sheep, have been found useful for screening purposes[16,17,38,46]. The species used (ruminants vs. monogastric animals) and the route of administration (oral vs. parenteral) may markedly affect the estimate of relative potency obtained, and due regard must also be paid to low solubility of the isoflavones and coumestans in neutral aqueous media.

Paper- thin-layer and gas-chromatographic micromethods are available for the separation of all the known phyto-estrogens from plant material, body fluids and animal tissue[10,28,41,87] and for their quantitative determination by spectrophotometry and fluorometry[10,41], flame-ionization detection[41] or receptor radioassay[71]. Another feasible approach is radioimmunoassay[7]. Receptor radioassay will not distinguish between the endogenous mammalian estrogens and nonsteroidal phyto-estrogens or stilboestrol, and will not detect the methylated herbal pro-estrogens. Immune sera, generated with isoflavone or coumestan haptens covalently attached to a protein carrier, will discriminate between steroidal and isoflavone-derived estrogens, and can be used to measure the active phenols as well as their *O*-methyl ethers[7].

9. Control measures

Control measures under investigation include pasture management designed to preserve a favourable balance between estrogenic legumes and grass[3,10,46,62]; measures to limit fungal infection of forage plants[12]; selection of isoflavone deficient clover mutants[29], which should have desirable agronomic properties, such as the ability to compete with the wild type in the field; and active vaccination with synthetic isoflavone derivatives coupled covalently to polypeptide carriers[7]. High titres of specific antibodies to phyto-estrogens are maintained in sheep for more than a year after primary immunization, are transferred to lambs through the colostrum and do not interfere with the action of endogenous hormones or breeding performance[24]. The protective value and economic feasibility of such vaccination remains to be established, and a more acceptable adjuvant than CFA, which is currently used, may have to be found. If effective, this method would provide the first example of immunization against a naturally occuring disease with a fully synthetic antigen, and as such could be of more general interest.

10. Concluding remarks

Estrogenic substances are widely distributed among plants serving as animal fodder or grown for direct human consumption. By and large they are nonsteroidal compounds capable of interacting directly with the cytoplasmic estrogen receptor by virtue of a structural resemblance to estradiol, – or apt to be metabolized to such estrogenomimetic compounds. It is likely that these compounds possess anabolic activity, but this aspect needs more critical documentation, preferrably including trials with purified substances. In excessive amounts herbal estrogens clearly have adverse effects on reproductive performance in sheep, and more sporadically in other ruminants. Some control measures under active investigation were briefly discussed.

Residues of these estrogens may accumulate in the carcass, and methods are available for their detection, but there is no information to suggest that they present a serious health hazard to man. The ubiquitous background of herbal and endogenous estrogens in animal

products may have to be considered when designing regulatory measures and forensic assay procedures to control the use of synthetic anabolic agents. Furthermore, the possible presence of phyto-estrogens in the control diet of experimental animals should be kept in mind when examining the effectiveness of other anabolic agents.

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